CHANGING THE HORIZON OF ENDOCRINE THERAPY RESISTANCE

EZH2 AND CIRCULATING TUMOR CELLS

ESTHER A. REIJM

Changing the Horizon of Endocrine Therapy Resistance

EZH2 and circulating tumor cells

Esther A. Reijm

The studies described in this thesis were performed within the framework of the Erasmus Postgraduate School Molecular Medicine at the Department of Medical Oncology and Cancer Genomics Netherlands, Erasmus MC – Cancer Institute, Erasmus University Medical Center, Rotterdam, The Netherlands.

The research projects were supported in part by TI Pharma, Leiden, The Netherlands, project T3-108 and T3-502 and the Gratama Stichting, Harlingen, The Netherlands.

Publication of this thesis was financially supported by kind contributions from:
Department of Medical Oncology of the Erasmus MC Cancer Institute, Erasmus University Rotterdam, Amphia Hospital Breda, ChipSoft BV, Pfizer BV, GR Instruments BV, and AstraZeneca BV (unrestricted grant).

ISBN: 978-94-028-0391-4

Cover photo by Vincent Fennis (www.vincentfennis.com)
Cover design and lay-out: Mirjam Leppers, Persoonlijk Proefschrift.nl
Printing: Ipskamp drukkers, Enschede

Changing the Horizon of Endocrine Therapy Resistance

EZH2 and circulating tumor cells

Nieuwe inzichten rondom endocriene therapie resistentie

EZH2 en circulerende tumorcellen

Proefschrift

ter verkrijging van de graad van doctor aan de

Erasmus Universiteit Rotterdam

op gezag van de rector magnificus

Prof.dr. H.A.P. Pols

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op

vrijdag 25 november 2016 om 9.30 uur

door

Esther Anneke Reijm

geboren te Hendrik-Ido-Ambacht

Erasmus University Rotterdam

PROMOTIECOMMISSIE

Promotoren:	Prof.dr. S. Sleijfer
	Prof.dr. P.M.J.J. Berns
Overige leden:	Prof.dr. I.P. Touw
	Prof.dr. F.J. van Kemenade
	Prof.dr. S.C. Linn

You don't need to be ill

to become better

CONTENTS

CHAPTER 1

General introduction and outline of the thesis

GENERAL INTRODUCTION AND OUTLINE OF THE THESIS

BREAST CANCER

Over the past years cancer has become a major public health issue being the most common cause of death in the Netherlands (1). In The Netherlands, the incidence rises with 3% per year and comparing incidences of 1989 (56,000) to 2011 (100,600) leads to the conclusion that the incidence has been doubled over the past 20 years (www.iknl.nl). For 2020, it is expected that 123,000 new cases of cancer will be diagnosed. Opposite to the increase in incidence, a decrease in mortality has been observed with half of the patients being alive at 5 years after establishing the diagnosis. This decrease is, amongst others, due to early detection but also to the availability of more, and more effective, treatment options (www.iknl.nl). However, mortality rates and the extent of possible therapies is still very different between the various types of cancer.

From all types of cancer, breast cancer is the most common cancer in females. Yearly, 14,000 new cases are diagnosed in The Netherlands. Of these women, 25% is younger than 50 years, 58% between 50 and 75 and 19% older than 75 years. Most of the cases are low stage breast cancer with favorable prognosis. Overall, 97% of the patients is still alive one year after diagnosis and the 10-year survival rate is currently 76% (www.iknl.nl). These relatively favorable statistics compared to other types of cancer is presumably partly due to the extensive screening program although the exact advantages of this program are still under debate.

PRIMARY BREAST CANCER

The prognosis of primary breast cancer is dependent on stage at presentation, which encompasses tumor size, lymph node status and presence or absence of distant metastases. Besides staging and grading, the molecular classification of tumors is an important prognostic and predictive factor. The most known molecular factors are the hormonal receptor molecules estrogen receptor (ER) and progesterone receptor (PR). The majority of breast tumors is positive for one or both of these receptors.
PR depends on progesterone stimulation and ER on estrogen for their growth. This way, ER is involved in (ab)normal cell growth and therefore forms an attractive and effective treatment target. The expression of ER and PR can be determined by multiple techniques, of which immunohistochemistry (IHC) is currently being used in the clinic. The presence of these receptors is inversely related with stage and histological grade since ER/PR-positive tumors are most commonly of low grade and well-differentiated (2).

Over the past years, many therapies have been developed that target the ER and have dramatically improved the prognosis of breast cancer. Tamoxifen, a selective estrogen receptor modulator, competes with estradiol for the binding to ER and represses the transcriptional activity of ER by inducing a conformational change of the receptor (3). For more than 30 years, tamoxifen forms the mainstay of treatment for premenopausal women and has been shown to safely reduce risks of breast cancer recurrence and death when used in the adjuvant setting (4).

An alternative strategy in treating ER-positive breast cancer was provided by the development of inhibitors of aromatase (AI). AI therapy depletes serum estradiol levels by inhibiting the aromatase enzyme that is involved in the synthesis of estradiol. It is mostly effective in postmenopausal women or in premenopausal women in whom ovarian function has been suppressed or the ovaries have been removed (5). The development of AI therapy led to a change in the endocrine therapy of postmenopausal women since several randomized controlled trials had shown that AI therapy compared to tamoxifen significantly improved disease-free survival (DFS). Nowadays, the use of AI therapy in a sequential manner with tamoxifen is the standard of care in postmenopausal ER-positive breast cancer patients (5).

Next to ER and PR, the human epidermal growth factor receptor 2 (HER2) is nowadays routinely measured in the clinic by using IHC and *in-situ* hybdrization (ISH) techniques. The *ERBB2* oncogene that encodes for the HER2 protein is amplified in 15% of the breast cancers and is associated with increased tumor aggressiveness and higher recurrence rates. It serves as a molecular target for specific therapies such as trastuzumab (6).

Additionally to ER, PR and HER2 characterization, over the years, large-scale genomic studies have been performed, which showed that breast cancer can be classified into several subtypes that demand to be treated differently due to their specific features and major differences in prognosis (7-9). The vast majority of all breast tumors (75%) are of the luminal subtype in which tumor cell proliferation is thought to depend on activity of the ER.
Breast cancer subtypes also differ by the pattern of metastatic disease. The most common metastatic site is bone, except for basal-like tumors which are more likely to metastasize to brain, lung and distant nodes. The most common metastatic sites in luminal HER2-positive and HER2-positive/ER-negative tumors are, next to bone, brain, liver and lung metastases (10).

METASTATIC BREAST CANCER

Approximately 5-10% of the patients present with distant metastases at the initial diagnosis. Additionally, approximately 30% of the patients initially diagnosed and treated for non-metastatic disease, depending on stage, develop distant metastases during follow-up despite prior intensive treatment (11). Metastatic breast cancer (MBC) is a highly heterogeneous disease for which many therapies have been developed over the years. Also here, treatment is highly dependent on the ER/PR/HER2 status of the tumor.

For most patients with ER/PR-positive disease, endocrine therapy is still the mainstay of treatment because of the favorable toxicity profile. In postmenopausal women, tamoxifen used to be the standard of care but clinical data have shown that AI therapy provides more benefit in terms of progression-free survival (PFS) and toxicity profile compared to tamoxifen (12, 13). For premenopausal women with ER-positive breast cancer, tamoxifen is still, after all those years, one of the standards of care. Key in treatment of

premenopausal women with metastatic ER/PR-positive disease is suppression of ovarian function for which luteinizing hormone-releasing hormone (LHRH) agonists are used. The combination of an LHRH agonist with tamoxifen has shown to significantly improve both PFS and overall survival (OS) compared to LHRH agonists alone (14).
For previously untreated ER-positive MBC, objective response rates of 32-44% and time to progression (TTP) of 8-10 months are observed when treated with endocrine therapy (15). In patients with ER/PR-positive disease without wide-spread metastatic disease, liver metastases, or lymphangitis pneumonia requiring a rapid tumor response, it is common practice to use multiple different hormonal agents in sequence as disease progresses. In postmenopausal women, when disease has progressed after non-steroidal AI therapy (anastrozole, letrozole) a steroidal aromatase inactivator (exemestane) can be used, nowadays combined with everolimus (see below) (16, 17). Another active agent is fulvestrant, an antiestrogen that binds to the ER and thus destabilizes the receptor resulting in its degradation. It has only estrogen antagonistic activity (18). Notably, the optimal sequence of the different endocrine therapy agents remains yet unknown.

Currently, the treatment of choice for HER2-positive tumors consists of a combination of blocking the function of HER2 together with chemotherapy. Treatment with trastuzumab, a humanized recombinant monoclonal antibody that blocks HER2-mediated signaling pathways results in a significantly longer TTP and survival compared to chemotherapy alone (19). Recently, it was found that the combination of antibodies against HER2, trastuzumab and pertuzumab, added to docetaxel resulted in a significantly improved OS compared to the combination of trastuzumab and docetaxel alone (20). This effect is caused by a more efficient signaling blockade since both antibodies target different extracellular regions of the HER2 protein (21). As a result, this triple therapy has now become the first-line standard approach for HER2-positive MBC.

Additionally, combined with HER2-targeting agents in HER2-positive MBC, chemotherapy is also required when there is extensive visceral organ involvement and there is a high need for a rapid response at diagnosis of ER+ MBC or when endocrine therapy has failed. Also for the subset of MBC patients where the tumor does not display ER/PR/HER2 expression, so called triple negative disease, chemotherapy is indicated. Taxanes and anthracyclines are mostly used but there are many chemotherapeutic agents with different mechanisms of actions and side-effects available for MBC, including capecitabine (antimetabolite, fluorouracil pro-drug), vinorelbine (vinca alkaloid), cis- and carboplatin (platinum analogs), mitomycin, eribulin, and etoposide (topoisomerase inhibitor) (15).

Despite of all the developments and enormous progress in the understanding of the biological behavior, MBC is still an incurable disease due to occurrence of resistance to therapy.

ENDOCRINE THERAPY RESISTANCE

As mentioned, endocrine treatment is one of the most important treatments used for MBC patients. Although many patients greatly benefit from endocrine therapies, a major limitation is that approximately 30% of the MBC patients never respond due to *de novo* resistance while all initial responders eventually relapse and develop progressive disease due to acquired resistance (22).

Resistance to single-agent cancer therapeutics is frequently the result of reactivation of the signaling pathway that is being targeted, indicating the impossibility of fully blocking the cancer-relevant signaling pathway. Already numerous factors accounting for resistance to endocrine therapy have been revealed and have led to the development of new drugs and new combinations. Loss of ER expression due to clonal selection of ER-negative cells or transcriptional repression of ER gene expression are some of the most known causes of resistance (23-25). It has been shown that demethylating agents or histone deacetylase inhibitors can reactivate ER expression when its promotor has been methylated leading to renewed sensitivity to endocrine therapies (26).
Another possible mechanism of acquired endocrine therapy resistance is the occurrence of mutations in *ESR1*, the gene coding for ER, during endocrine therapy. It has been shown that activating *ESR1* mutations are frequently present in ER-positive metastases that have become (AI) resistant but are absent in the corresponding primary tumors (27-30).

Next, *in vitro* experiments showed that overexpression of the HER2 receptor leads to activation of MAPK and inhibition of ER expression (31, 32), offering a rationale for dual blockade of HER2 and ER in order to overcome resistance (33). Furthermore, some tumors that are originally negative for HER2 show HER2 amplification after tumor progression during tamoxifen therapy (34).

The PI3K/AKT/mTOR pathway is the most frequently activated signaling pathway in breast cancer and emerging evidence points to hyperactivation of this pathway as a key mechanism of endocrine therapy resistance (35, 36). The combination of an mTOR inhibitor (everolimus) and AI therapy (exemestane) has already been extensively studied and set as golden standard in the treatment of MBC patients who failed previous treatment with a non-steriodal AI due to its prolonged PFS compared to exemestane alone (17). Although the combination therapy of everolimus and exemestane resulted in a clinically meaningful improvement in PFS, it has recently been shown that OS was not significantly improved (37). Several other agents that target this pathway are currently under investigation and have shown promising results (38).

Importantly, although much is already known, there is still a high need to get more insight into the factors causing resistance to endocrine therapy in order to develop methods to overcome resistance and to identify biomarkers that can contribute to a more personalized treatment approach of individual patients.

EZH2

One of the more recent discovered factors that might contribute to endocrine therapy resistance is Enhancer of Zeste Homolog 2 (EZH2). After a genome-wide profiling study in MBC patients, the Enhancer of Zeste family was revealed as one of the involved factors (39).

EZH2 is one of the Polycomb group (PcG) proteins and forms the catalytic subunit of the Polycomb repressive complex 2 (PRC2). It acts as a histone methyltransferase that mediates trimethylation of lysine residue 27 on histone 3 (H3K27) in order to control transcriptional processes. The H3K27me3 activity is dependent on post-translational modifications. For example, phosphorylation at serine 21 of EZH2 by Akt1 reduces its activity while phosphorylation at threonine 350 by CDK1 and CDK2 is required to maintain the H3K27me3 repressive marks on its target genes (40, 41). By repressing certain genes, EZH2 plays a pivotal role in embryonic development, cell differentiation, and carcinogenesis (42-44). Evidence has been provided that EZH2 might play a role in the development of many types of cancers including prostate cancer (45), lymphomas (46, 47), urogenital tract tumors (48-50), and breast cancer (51).

Increased expression of EZH2 has been associated with increased tumor cell proliferation and therefore worse survival (48, 51-53). The association between EZH2 and tumor aggressiveness has been confirmed in several tumor types (54-56) and resulted in the consideration of EZH2 as an important therapeutic target. Two microRNAs (miRs) able to regulate EZH2 expression in different tissues have been discovered, i.e. miR26a and miR-101 (57, 58).

Since the great impact EZH2 inhibition might have in the treatment of multiple types of tumors, synthetic inhibitors are of more interest. 3-deazaneplanocin A (DZNep) can inhibit H3K27me3 and is an effective method to revert epithelial-to-mesenchymal transition (EMT), making it an interesting agent to prevent metastatic disease (59). However, this agent is a global histone methyltransferase inhibitor and not specific for EZH2 (60). Fortunately, small-molecule inhibitors of EZH2 have been developed and have shown to be able to decrease the histone methyltransferase activity of EZH2 resulting in reactivation of silenced target genes, especially in lymphomas with EZH2-activating mutations (61, 62). However, direct EZH2 inhibition by these small molecules may have an unfavorable effect on global methylation patterns in non-cancerous cells. Affecting the EZH2 phosphorylation pattern through inhibitors of CDK1/2 can diminish the EZH2 activity in tumor cells and may be of more interest since it less affects the global EZH2 mediated gene silencing (41).

More knowledge is however needed to identify the exact role of EZH2 in endocrine therapy resistance in breast cancer and to what extent.

CIRCULATING TUMOR CELLS

Another reason underlying resistance in MBC is the constantly changing molecular make-up of tumor cells. Currently, the determination of predictive factors for treatment decision making is most commonly done in the primary tumor tissue while differences in clinically relevant molecular characteristics between the primary tumor and metastases are increasingly recognized. Characteristics of metastatic cells rather than of the primary tumor are therefore likely to be more important since the ultimate outcome of cancer patients is determined by the behavior of metastases. With respect to the expression of ER, tumor status differs in 20% when comparing the primary tumor with its metastases, which would lead to a relevant treatment change in a substantial number of patients (25, 63, 64). This change in tumor status can be partly explained by the fact that tumors consist of different subclones with differential gene expression and diverse abilities to metastasize. Moreover, over the course of tumor development and exposure to treatment, genomic instability leads to different genomic landscapes that can cause differences in response to treatment over time (65). As also mentioned above, one of the key mechanisms in acquired endocrine therapy resistance is the occurrence of activating mutations in *ESR1* in metastatic tumors during treatment causing proliferation in the absence of hormone (28, 29).

Therefore, repetitive analyses of metastatic tissue could probably lead to more appropriate information for response prediction. However, metastatic tissue is often hard to obtain and only possible through invasive procedures, which limits its use in clinical practice. Hence, the characterization of circulating tumor cells (CTCs) poses an attractive alternative. CTCs are tumor cells found in the peripheral blood and are thought to originate from either the primary tumor or metastases. They can be obtained through venipunctures and can serve as a 'liquid biopsy'.

The detection of CTCs is challenging with a median CTC count of 3 - 5 per 7.5 mL of blood in MBC patients (66). Detection methods therefore require extreme sensitivity and specificity. Over the years, many different CTC detection methods have been developed based on different properties of CTCs that distinguish them from normal blood cells (67). Some techniques isolate CTCs on physical properties (size, density, electric charges) but most of them use their biological properties by using surface protein expression, which is mostly the epithelial marker EpCAM. Amongst these methods, the CellSearch system is the only FDA-cleared system. Major drawback with epithelial marker based techniques however is that cells that have undergone EMT, thereby potentially losing EpCAM expression, possibly remain undetected whilst these cells are thought to underlie haematogenous metastases and therefore associate with poor prognosis (68-71). Methods using expression of mesenchymal markers like N-Cadherin and Vimentin that are not repressed during EMT are under development to overcome the problem of missing this putatively most aggressive CTC subpopulation (72, 73). However, it has recently been demonstrated that the CellSearch system is able to detect CTCs in patients with triple negative breast cancer although the sensitivity is slightly lower than in non-triple negative disease (74).

Despite the technical advancements in CTC detection, CTC enumeration is not yet ready to be implemented as a cancer screening tool or to be used for diagnosis. However, enumeration of CTCs has repeatedly proven to have prognostic value in multiple tumor types where the presence of CTCs in breast cancer has shown to be associated with a worse survival (75-79).

Besides detection of CTCs a major clinical challenge encompasses characterizing CTCs in order to base clinical decision making more on the characteristics of metastatic cells. However, since the isolation of pure fractions of CTCs is a major technological challenge, molecular characterization of CTCs is difficult to achieve since its low frequency and presence amongst a substantial number of leukocytes. It is however worthwhile to strive to since it holds great promise in improving knowledge on mechanisms underlying resistance to endocrine therapy. Nowadays, several techniques have been developed to molecularly characterize CTCs for drug target expression (77, 80, 81), mutations (82), and gene expression (83-85) and has, amongst others, revealed the high frequency of *PIK3CA* mutations in CTCs, which offers rationale for exploring PI3K-inhibitors in these patients (86).

Several clinical studies are now being conducted in order to better understand the possible use of both the count and characteristics of CTCs in the clinic. Most of them are focusing on the possibility to use CTC counts to test for early chemoresistance or to assess new treatment strategies using anti-HER2 agents (87).

Concerning the use of CTCs in better understanding endocrine therapy resistance not much is yet known. It has been demonstrated that CTCs of MBC patients with ER-positive primary tumors frequently lack ER expression, which is a possible explanation for endocrine therapy resistance. In these CTCs, *ESR1* mutations were not found (88). Currently, the clinical implication of a CTC-Endocrine Therapy Index (CTC-ETI), in which the protein expression of markers that play a role in endocrine therapy response (e.g. ER, B-cell lymphoma 2 (BCL-2), HER2, and Ki67) are stained in CTCs, is evaluated in a prospective trial (89). But more research is needed to fully understand whether or not CTC characterization can explain endocrine therapy resistance and can provide new guidance to overcome resistance.

AIMS AND OUTLINE OF THE THESIS

This thesis is aimed at exploring putative factors that are involved in endocrine therapy resistance by using cell line models and both primary tumors and CTCs of patients. The contribution of EZH2 expression in the behavior and drug response of tumors has been investigated on multiple levels of expression and the importance of characterization of CTCs has been explored.

In **Chapter 2** we evaluate the association between different mRNA expression levels of EZH2 and PFS for ER-positive breast cancer patients who received first-line tamoxifen for advanced disease. In this chapter we propose the inverse relation between EZH2 and ER and suggest EZH2 as a potential treatment target to increase the anti-tumor activity of anti-estrogen therapies in breast cancer.

In order to obtain more understanding about the molecular pathway of EZH2, we performed pathway analysis on microarray data of 65 ER-positive tumors as being described in **Chapter 3**. By using expression levels of miR-26a and miR-101, both having EZH2 as common target, the goal was to discover a pathway that explains the association between high levels of miR-26a and low levels of EZH2 in order to define more factors that are related with a favorable outcome to tamoxifen therapy.

In **Chapter 4** we further explore the association between EZH2 and response to tamoxifen therapy by using a tissue microarray (TMA) created of ER-positive primary breast tumor tissues of 250 MBC patients who received tamoxifen as first-line therapy. EZH2 expression can be scored by IHC for intensity and quantity, and can be related to PFS.

Since nowadays AIs form the major first-line treatments for MBC, the next chapters contain studies on resistance against this type of therapy. In **Chapter 5** we combine both ER and EZH2 by investigating the epigenetic modification H3K27me3 as a result of EZH2 activity on ER and check for its consequences on treatment outcome for first-line AI treatment.

In addition to the results obtained in cell line models and primary tumors of patients, we performed studies on CTCs of patients with advanced breast cancer. In **Chapter 6** we use CTC characterization of MBC patients treated with endocrine therapy to investigate genes that predict response to AI therapy to guide clinical decision making in a more valuable way since the decision would be based on recent tumor events by using CTCs instead of the primary tumor that has been removed years before.

In conclusion, this thesis entitled *'Changing the horizon of endocrine therapy resistance'* explores the factors involved in endocrine therapy resistance aiming to come to a more personalized and targeted anti-cancer treatment.

REFERENCES

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. CA Cancer J Clin. 2015;65(1):5-29.
2. Dunnwald LK, Rossing MA, Li CI. Hormone receptor status, tumor characteristics, and prognosis: a prospective cohort of breast cancer patients. Breast Cancer Res. 2007;9(1):R6.
3. Orlando L, Schiavone P, Fedele P, Calvani N, Nacci A, Rizzo P, et al. Molecularly targeted endocrine therapies for breast cancer. Cancer Treat Rev. 2010;36 Suppl 3:S67-71.
4. Early Breast Cancer Trialists' Collaborative G, Davies C, Godwin J, Gray R, Clarke M, Cutter D, et al. Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. Lancet. 2011;378(9793):771-84.
5. Schiavon G, Smith IE. Status of adjuvant endocrine therapy for breast cancer. Breast Cancer Res. 2014;16(2):206.
6. Sauter G, Lee J, Bartlett JM, Slamon DJ, Press MF. Guidelines for human epidermal growth factor receptor 2 testing: biologic and methodologic considerations. J Clin Oncol. 2009;27(8):1323-33.
7. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. Nature. 2000;406(6797):747-52.
8. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A. 2001;98(19):10869-74.
9. Herschkowitz JI, Simin K, Weigman VJ, Mikaelian I, Usary J, Hu Z, et al. Identification of conserved gene expression features between murine mammary carcinoma models and human breast tumors. Genome Biol. 2007;8(5):R76.
10. Smid M, Wang Y, Zhang Y, Sieuwerts AM, Yu J, Klijn JG, et al. Subtypes of breast cancer show preferential site of relapse. Cancer Res. 2008;68(9):3108-14.
11. Cardoso F, Harbeck N, Fallowfield L, Kyriakides S, Senkus E, Group EGW. Locally recurrent or metastatic breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol. 2012;23 Suppl 7:vii11-9.
12. Cardoso F, Bischoff J, Brain E, Zotano AG, Luck HJ, Tjan-Heijnen VC, et al. A review of the treatment of endocrine responsive metastatic breast cancer in postmenopausal women. Cancer Treat Rev. 2013;39(5):457-65.
13. Droog M, Beelen K, Linn S, Zwart W. Tamoxifen resistance: from bench to bedside. Eur J Pharmacol. 2013;717(1-3):47-57.
14. Klijn JG, Blamey RW, Boccardo F, Tominaga T, Duchateau L, Sylvester R, et al. Combined tamoxifen and luteinizing hormone-releasing hormone (LHRH) agonist versus LHRH agonist alone in premenopausal advanced breast cancer: a meta-analysis of four randomized trials. J Clin Oncol. 2001;19(2):343-53.
15. Amar S, Roy V, Perez EA. Treatment of metastatic breast cancer: looking towards the future. Breast Cancer Res Treat. 2009;114(3):413-22.
16. Lonning PE, Bajetta E, Murray R, Tubiana-Hulin M, Eisenberg PD, Mickiewicz E, et al. Activity of exemestane in metastatic breast cancer after failure of nonsteroidal aromatase inhibitors: a phase II trial. J Clin Oncol. 2000;18(11):2234-44.

17. Baselga J, Campone M, Piccart M, Burris HA, 3rd, Rugo HS, Sahmoud T, et al. Everolimus in postmenopausal hormone-receptor-positive advanced breast cancer. N Engl J Med. 2012;366(6):520-9.
18. Ingle JN, Suman VJ, Rowland KM, Mirchandani D, Bernath AM, Camoriano JK, et al. Fulvestrant in women with advanced breast cancer after progression on prior aromatase inhibitor therapy: North Central Cancer Treatment Group Trial N0032. J Clin Oncol. 2006;24(7):1052-6.
19. Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med. 2001;344(11):783-92.
20. Swain SM, Baselga J, Kim SB, Ro J, Semiglazov V, Campone M, et al. Pertuzumab, trastuzumab, and docetaxel in HER2-positive metastatic breast cancer. N Engl J Med. 2015;372(8):724-34.
21. Nahta R, Hung MC, Esteva FJ. The HER-2-targeting antibodies trastuzumab and pertuzumab synergistically inhibit the survival of breast cancer cells. Cancer Res. 2004;64(7):2343-6.
22. Early Breast Cancer Trialists' Collaborative G. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet. 2005;365(9472):1687-717.
23. Amir E, Miller N, Geddie W, Freedman O, Kassam F, Simmons C, et al. Prospective study evaluating the impact of tissue confirmation of metastatic disease in patients with breast cancer. J Clin Oncol. 2012;30(6):587-92.
24. Gong Y, Han EY, Guo M, Pusztai L, Sneige N. Stability of estrogen receptor status in breast carcinoma: a comparison between primary and metastatic tumors with regard to disease course and intervening systemic therapy. Cancer. 2011;117(4):705-13.
25. Thompson AM, Jordan LB, Quinlan P, Anderson E, Skene A, Dewar JA, et al. Prospective comparison of switches in biomarker status between primary and recurrent breast cancer: the Breast Recurrence In Tissues Study (BRITS). Breast Cancer Res. 2010;12(6):R92.
26. Ferguson AT, Lapidus RG, Baylin SB, Davidson NE. Demethylation of the estrogen receptor gene in estrogen receptor-negative breast cancer cells can reactivate estrogen receptor gene expression. Cancer Res. 1995;55(11):2279-83.
27. Jeselsohn R, Yelensky R, Buchwalter G, Frampton G, Meric-Bernstam F, Gonzalez-Angulo AM, et al. Emergence of constitutively active estrogen receptor-alpha mutations in pretreated advanced estrogen receptor-positive breast cancer. Clin Cancer Res. 2014;20(7):1757-67.
28. Robinson DR, Wu YM, Vats P, Su F, Lonigro RJ, Cao X, et al. Activating ESR1 mutations in hormone-resistant metastatic breast cancer. Nat Genet. 2013;45(12):1446-51.
29. Toy W, Shen Y, Won H, Green B, Sakr RA, Will M, et al. ESR1 ligand-binding domain mutations in hormone-resistant breast cancer. Nat Genet. 2013;45(12):1439-45.
30. Jordan VC, Curpan R, Maximov PY. Estrogen receptor mutations found in breast cancer metastases integrated with the molecular pharmacology of selective ER modulators. J Natl Cancer Inst. 2015;107(6):djv075.
31. Creighton CJ, Hilger AM, Murthy S, Rae JM, Chinnaiyan AM, El-Ashry D. Activation of mitogen-activated protein kinase in estrogen receptor alpha-positive breast cancer cells in vitro induces an in vivo molecular phenotype of estrogen receptor alpha-negative

human breast tumors. Cancer Res. 2006;66(7):3903-11.
32. Pancholi S, Lykkesfeldt AE, Hilmi C, Banerjee S, Leary A, Drury S, et al. ERBB2 influences the subcellular localization of the estrogen receptor in tamoxifen-resistant MCF-7 cells leading to the activation of AKT and RPS6KA2. Endocr Relat Cancer. 2008;15(4):985-1002.
33. Leary AF, Drury S, Detre S, Pancholi S, Lykkesfeldt AE, Martin LA, et al. Lapatinib restores hormone sensitivity with differential effects on estrogen receptor signaling in cell models of human epidermal growth factor receptor 2-negative breast cancer with acquired endocrine resistance. Clin Cancer Res. 2010;16(5):1486-97.
34. Gutierrez MC, Detre S, Johnston S, Mohsin SK, Shou J, Allred DC, et al. Molecular changes in tamoxifen-resistant breast cancer: relationship between estrogen receptor, HER-2, and p38 mitogen-activated protein kinase. J Clin Oncol. 2005;23(11):2469-76.
35. Cavazzoni A, Bonelli MA, Fumarola C, La Monica S, Airoud K, Bertoni R, et al. Overcoming acquired resistance to letrozole by targeting the PI3K/AKT/mTOR pathway in breast cancer cell clones. Cancer Lett. 2012;323(1):77-87.
36. Miller TW, Balko JM, Arteaga CL. Phosphatidylinositol 3-kinase and antiestrogen resistance in breast cancer. J Clin Oncol. 2011;29(33):4452-61.
37. Piccart M, Hortobagyi GN, Campone M, Pritchard KI, Lebrun F, Ito Y, et al. Everolimus plus exemestane for hormone-receptor-positive, human epidermal growth factor receptor-2-negative advanced breast cancer: overall survival results from BOLERO-2dagger. Ann Oncol. 2014;25(12):2357-62.
38. Ciruelos Gil EM. Targeting the PI3K/AKT/mTOR pathway in estrogen receptor-positive breast cancer. Cancer Treat Rev. 2014;40(7):862-71.
39. Jansen MP, Foekens JA, van Staveren IL, Dirkzwager-Kiel MM, Ritstier K, Look MP, et al. Molecular classification of tamoxifen-resistant breast carcinomas by gene expression profiling. J Clin Oncol. 2005;23(4):732-40.
40. Cha TL, Zhou BP, Xia W, Wu Y, Yang CC, Chen CT, et al. Akt-mediated phosphorylation of EZH2 suppresses methylation of lysine 27 in histone H3. Science. 2005;310(5746):306-10.
41. Chen S, Bohrer LR, Rai AN, Pan Y, Gan L, Zhou X, et al. Cyclin-dependent kinases regulate epigenetic gene silencing through phosphorylation of EZH2. Nat Cell Biol. 2010;12(11):1108-14.
42. Bracken AP, Dietrich N, Pasini D, Hansen KH, Helin K. Genome-wide mapping of Polycomb target genes unravels their roles in cell fate transitions. Genes Dev. 2006;20(9):1123-36.
43. Cao R, Wang L, Wang H, Xia L, Erdjument-Bromage H, Tempst P, et al. Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science. 2002;298(5595):1039-43.
44. Schuettengruber B, Chourrout D, Vervoort M, Leblanc B, Cavalli G. Genome regulation by polycomb and trithorax proteins. Cell. 2007;128(4):735-45.
45. Varambally S, Dhanasekaran SM, Zhou M, Barrette TR, Kumar-Sinha C, Sanda MG, et al. The polycomb group protein EZH2 is involved in progression of prostate cancer. Nature. 2002;419(6907):624-9.
46. Raaphorst FM, van Kemenade FJ, Blokzijl T, Fieret E, Hamer KM, Satijn DP, et al. Coexpression of BMI-1 and EZH2 polycomb group genes in Reed-Sternberg cells of

Hodgkin's disease. Am J Pathol. 2000;157(3):709-15.
47. Visser HP, Gunster MJ, Kluin-Nelemans HC, Manders EM, Raaphorst FM, Meijer CJ, et al. The Polycomb group protein EZH2 is upregulated in proliferating, cultured human mantle cell lymphoma. Br J Haematol. 2001;112(4):950-8.
48. Bachmann IM, Halvorsen OJ, Collett K, Stefansson IM, Straume O, Haukaas SA, et al. EZH2 expression is associated with high proliferation rate and aggressive tumor subgroups in cutaneous melanoma and cancers of the endometrium, prostate, and breast. J Clin Oncol. 2006;24(2):268-73.
49. Raman JD, Mongan NP, Tickoo SK, Boorjian SA, Scherr DS, Gudas LJ. Increased expression of the polycomb group gene, EZH2, in transitional cell carcinoma of the bladder. Clin Cancer Res. 2005;11(24 Pt 1):8570-6.
50. Weikert S, Christoph F, Kollermann J, Muller M, Schrader M, Miller K, et al. Expression levels of the EZH2 polycomb transcriptional repressor correlate with aggressiveness and invasive potential of bladder carcinomas. Int J Mol Med. 2005;16(2):349-53.
51. Kleer CG, Cao Q, Varambally S, Shen R, Ota I, Tomlins SA, et al. EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells. Proc Natl Acad Sci U S A. 2003;100(20):11606-11.
52. Collett K, Eide GE, Arnes J, Stefansson IM, Eide J, Braaten A, et al. Expression of enhancer of zeste homologue 2 is significantly associated with increased tumor cell proliferation and is a marker of aggressive breast cancer. Clin Cancer Res. 2006;12(4):1168-74.
53. Raaphorst FM, Meijer CJ, Fieret E, Blokzijl T, Mommers E, Buerger H, et al. Poorly differentiated breast carcinoma is associated with increased expression of the human polycomb group EZH2 gene. Neoplasia. 2003;5(6):481-8.
54. Eskander RN, Ji T, Huynh B, Wardeh R, Randall LM, Hoang B. Inhibition of enhancer of zeste homolog 2 (EZH2) expression is associated with decreased tumor cell proliferation, migration, and invasion in endometrial cancer cell lines. Int J Gynecol Cancer. 2013;23(6):997-1005.
55. Li H, Cai Q, Godwin AK, Zhang R. Enhancer of zeste homolog 2 promotes the proliferation and invasion of epithelial ovarian cancer cells. Mol Cancer Res. 2010;8(12):1610-8.
56. Ougolkov AV, Bilim VN, Billadeau DD. Regulation of pancreatic tumor cell proliferation and chemoresistance by the histone methyltransferase enhancer of zeste homologue 2. Clin Cancer Res. 2008;14(21):6790-6.
57. Varambally S, Cao Q, Mani RS, Shankar S, Wang X, Ateeq B, et al. Genomic loss of microRNA-101 leads to overexpression of histone methyltransferase EZH2 in cancer. Science. 2008;322(5908):1695-9.
58. Wong CF, Tellam RL. MicroRNA-26a targets the histone methyltransferase Enhancer of Zeste homolog 2 during myogenesis. J Biol Chem. 2008;283(15):9836-43.
59. Crea F, Paolicchi E, Marquez VE, Danesi R. Polycomb genes and cancer: time for clinical application? Crit Rev Oncol Hematol. 2012;83(2):184-93.
60. Miranda TB, Cortez CC, Yoo CB, Liang G, Abe M, Kelly TK, et al. DZNep is a global histone methylation inhibitor that reactivates developmental genes not silenced by DNA methylation. Mol Cancer Ther. 2009;8(6):1579-88.
61. McCabe MT, Ott HM, Ganji G, Korenchuk S, Thompson C, Van Aller GS, et al. EZH2 inhibition as a therapeutic strategy for lymphoma with EZH2-activating mutations.

Nature. 2012;492(7427):108-12.
62. Qi W, Chan H, Teng L, Li L, Chuai S, Zhang R, et al. Selective inhibition of Ezh2 by a small molecule inhibitor blocks tumor cells proliferation. Proc Natl Acad Sci U S A. 2012;109(52):21360-5.
63. Campbell PJ, Yachida S, Mudie LJ, Stephens PJ, Pleasance ED, Stebbings LA, et al. The patterns and dynamics of genomic instability in metastatic pancreatic cancer. Nature. 2010;467(7319):1109-13.
64. Xiao C, Gong Y, Han EY, Gonzalez-Angulo AM, Sneige N. Stability of HER2-positive status in breast carcinoma: a comparison between primary and paired metastatic tumors with regard to the possible impact of intervening trastuzumab treatment. Ann Oncol. 2011;22(7):1547-53.
65. Burrell RA, McGranahan N, Bartek J, Swanton C. The causes and consequences of genetic heterogeneity in cancer evolution. Nature. 2013;501(7467):338-45.
66. Allard WJ, Matera J, Miller MC, Repollet M, Connelly MC, Rao C, et al. Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. Clin Cancer Res. 2004;10(20):6897-904.
67. Toss A, Mu Z, Fernandez S, Cristofanilli M. CTC enumeration and characterization: moving toward personalized medicine. Ann Transl Med. 2014;2(11):108.
68. Munz M, Baeuerle PA, Gires O. The emerging role of EpCAM in cancer and stem cell signaling. Cancer Res. 2009;69(14):5627-9.
69. Willipinski-Stapelfeldt B, Riethdorf S, Assmann V, Woelfle U, Rau T, Sauter G, et al. Changes in cytoskeletal protein composition indicative of an epithelial-mesenchymal transition in human micrometastatic and primary breast carcinoma cells. Clin Cancer Res. 2005;11(22):8006-14.
70. Woelfle U, Sauter G, Santjer S, Brakenhoff R, Pantel K. Down-regulated expression of cytokeratin 18 promotes progression of human breast cancer. Clin Cancer Res. 2004;10(8):2670-4.
71. Sieuwerts AM, Kraan J, Bolt J, van der Spoel P, Elstrodt F, Schutte M, et al. Anti-epithelial cell adhesion molecule antibodies and the detection of circulating normal-like breast tumor cells. J Natl Cancer Inst. 2009;101(1):61-6.
72. Satelli A, Mitra A, Brownlee Z, Xia X, Bellister S, Overman MJ, et al. Epithelial-mesenchymal transitioned circulating tumor cells capture for detecting tumor progression. Clin Cancer Res. 2015;21(4):899-906.
73. Yu M, Bardia A, Wittner BS, Stott SL, Smas ME, Ting DT, et al. Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. Science. 2013;339(6119):580-4.
74. Paoletti C, Li Y, Muniz MC, Kidwell KM, Aung K, Thomas DG, et al. Significance of Circulating Tumor Cells in Metastatic Triple-Negative Breast Cancer Patients within a Randomized, Phase II Trial: TBCRC 019. Clin Cancer Res. 2015;21(12):2771-9.
75. Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med. 2004;351(8):781-91.

76. Cristofanilli M, Hayes DF, Budd GT, Ellis MJ, Stopeck A, Reuben JM, et al. Circulating tumor cells: a novel prognostic factor for newly diagnosed metastatic breast cancer. J Clin Oncol. 2005;23(7):1420-30.
77. Riethdorf S, Muller V, Zhang L, Rau T, Loibl S, Komor M, et al. Detection and HER2 expression of circulating tumor cells: prospective monitoring in breast cancer patients treated in the neoadjuvant GeparQuattro trial. Clin Cancer Res. 2010;16(9):2634-45.
78. Zhang L, Riethdorf S, Wu G, Wang T, Yang K, Peng G, et al. Meta-analysis of the prognostic value of circulating tumor cells in breast cancer. Clin Cancer Res. 2012;18(20):5701-10.
79. Bidard FC, Peeters DJ, Fehm T, Nole F, Gisbert-Criado R, Mavroudis D, et al. Clinical validity of circulating tumour cells in patients with metastatic breast cancer: a pooled analysis of individual patient data. Lancet Oncol. 2014;15(4):406-14.
80. Attard G, Swennenhuis JF, Olmos D, Reid AH, Vickers E, A'Hern R, et al. Characterization of ERG, AR and PTEN gene status in circulating tumor cells from patients with castration-resistant prostate cancer. Cancer Res. 2009;69(7):2912-8.
81. Fehm T, Muller V, Aktas B, Janni W, Schneeweiss A, Stickeler E, et al. HER2 status of circulating tumor cells in patients with metastatic breast cancer: a prospective, multicenter trial. Breast Cancer Res Treat. 2010;124(2):403-12.
82. Gasch C, Bauernhofer T, Pichler M, Langer-Freitag S, Reeh M, Seifert AM, et al. Heterogeneity of epidermal growth factor receptor status and mutations of KRAS/PIK3CA in circulating tumor cells of patients with colorectal cancer. Clin Chem. 2013;59(1):252-60.
83. Smirnov DA, Zweitzig DR, Foulk BW, Miller MC, Doyle GV, Pienta KJ, et al. Global gene expression profiling of circulating tumor cells. Cancer Res. 2005;65(12):4993-7.
84. Sieuwerts AM, Mostert B, Bolt-de Vries J, Peeters D, de Jongh FE, Stouthard JM, et al. mRNA and microRNA expression profiles in circulating tumor cells and primary tumors of metastatic breast cancer patients. Clin Cancer Res. 2011;17(11):3600-18.
85. Onstenk W, Gratama JW, Foekens JA, Sleijfer S. Towards a personalized breast cancer treatment approach guided by circulating tumor cell (CTC) characteristics. Cancer Treat Rev. 2013.
86. Markou A, Farkona S, Schiza C, Efstathiou T, Kounelis S, Malamos N, et al. PIK3CA mutational status in circulating tumor cells can change during disease recurrence or progression in patients with breast cancer. Clin Cancer Res. 2014;20(22):5823-34.
87. Bidard FC, Fehm T, Ignatiadis M, Smerage JB, Alix-Panabieres C, Janni W, et al. Clinical application of circulating tumor cells in breast cancer: overview of the current interventional trials. Cancer Metastasis Rev. 2013;32(1-2):179-88.
88. Babayan A, Hannemann J, Spotter J, Muller V, Pantel K, Joosse SA. Heterogeneity of estrogen receptor expression in circulating tumor cells from metastatic breast cancer patients. PLoS One. 2013;8(9):e75038.
89. Paoletti C, Muniz MC, Thomas DG, Griffith KA, Kidwell KM, Tokudome N, et al. Development of Circulating Tumor Cell-Endocrine Therapy Index in Patients with Hormone Receptor Positive Breast Cancer. Clin Cancer Res. 2014.

CHAPTER 2

Decreased expression of EZH2 is associated with upregulation of ER and favorable outcome to tamoxifen in advanced breast cancer

E.A. Reijm*, M.P.H.M. Jansen*, K. Ruigrok-Ritstier, I.L. van Staveren, M.P. Look, M.E. Meijer-van Gelder, A.M. Sieuwerts, S. Sleijfer, J.A. Foekens, E.M.J.J. Berns

Breast Cancer Res Treat 2011; 125(2): 387-394

* These authors contributed equally.

ABSTRACT

The purpose of this study is to investigate EZH2 in a large series of breast cancer patients for its prognostic and predictive value, and to evaluate its functional role in treatment response in vitro. EZH2 levels were measured using quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) in primary breast cancer specimens and related to clinicopathologic factors and disease outcome. EZH2 expression was downregulated with siRNAs in MCF7, to assess expression alterations of putative EZH2 downstream genes and to determine cell numbers after treatment with the anti-estrogen ICI 164384. In 688 lymph node-negative patients who did not receive adjuvant systemic therapy, EZH2 was not significantly correlated with metastasis-free survival (MFS). In 278 patients with advanced disease treated with first-line tamoxifen monotherapy, the tertile with highest EZH2 levels was associated with the lowest clinical benefit (OR = 0.48; $P = 0.02$) and with a shorter progression-free survival (PFS) in both univariate (HR = 1.80; $P < 0.001$) and multivariate analysis, including traditional factors (HR = 1.61; $P = 0.004$). In vitro, EZH2 silencing in MCF7 caused a 38% decrease in cell numbers ($P < 0.001$) whereas ICI 164384 treatment resulted in a 25% decrease ($P < 0.001$) compared to controls. Combining EZH2 silencing with ICI treatment reduced cell numbers with 67% ($P < 0.001$) compared to control conditions. EZH2 downregulation was associated with an almost two-fold upregulation of the estrogen receptor alpha (ER) ($P = 0.001$). In conclusion, EZH2 has no prognostic value in breast cancer. High levels of EZH2 are associated with poor outcome to tamoxifen therapy in advanced breast cancer. Downregulated EZH2 leads to upregulation of the ER and better response to anti-estrogens.

INTRODUCTION

The anti-estrogen tamoxifen has been used for treatment of estrogen receptor alpha (ER) positive breast cancer for more than 20 years both in the adjuvant and advanced setting. Although the majority of breast tumors express the ER, approximately half of the patients with ER-positive advanced disease do not respond to endocrine therapy or will eventually develop resistance. As a consequence, there is a high need for markers to identify patients likely to benefit from tamoxifen and to get a better insight into mechanisms conferring resistance.

In a previous genome-wide profiling study in breast cancer patients with advanced disease, we revealed an 81-gene signature for resistance to first-line tamoxifen treatment (1). One of the gene families from this profile is Enhancer of Zeste, consisting of Enhancer of Zeste Homolog 1 (EZH1, OMIM 601674) and Enhancer of Zeste Homolog 2 (EZH2, OMIM 601573). Both EZ homologs belong to Polycomb group (PcG) proteins, and are involved in transcriptional control and epigenetic memory maintenance for preservation of cellular characteristics (2). EZH2 comprises the core of the Polycomb Repressor Complex 2 (PRC2) (3-5), has histone lysine methyltransferase activity, and mediates di- and trimethylation on histone 3 lysine residue 27 (H3K27) (2). EZH1 can also be a part of PRC2, although with low histone lysine methyltransferase activity (6). We previously explored EZH1 with qRT-PCR, but did not observe a significant correlation with clinical outcome in 229 ER-positive tumors of patients with advanced disease treated with first-line tamoxifen monotherapy (7).

In contrast to EZH1, EZH2 has been extensively studied in malignancies. Increased expression of EZH2 in breast, prostate, and bladder cancer has been associated with a high histological grade and increased tumor cell proliferation (8-10). In addition, EZH2 was identified as an adverse prognostic marker for breast and prostate cancer, but these studies included only small series of patients (11, 12). Its predictive value for outcome to tamoxifen in advanced breast cancer has, however, not been studied yet.

The aims of this study were [1] to assess the prognostic value of EZH2 in a large series of patients, [2] to study its predictive value for outcome to tamoxifen treatment in advanced breast cancer, and [3] to explore its functional role in endocrine therapy resistance.

PATIENTS AND METHODS

PATIENTS

This retrospective study, in which coded tumor tissues were used, has been approved by the medical ethics committee of the Erasmus MC Rotterdam, The Netherlands (MEC 02.953), was performed in accordance with the Code of Conduct of the Federation of Medical Scientific Societies in the Netherlands (http://www.fmwv.nl), and reported following the REMARK recommendations (13), wherever possible. Frozen breast tumor tissue specimens of female patients with primary operable breast cancer who entered the clinic between 1979 and 1996 were analyzed. Follow-up, tumor staging, and response to therapy were defined by standard International Union Against Cancer (Geneva, Switzerland) classification criteria (14).

Tumor protein expression levels of ER and progesterone receptor (PgR) were determined (15), and 10 fmol/mg cytosolic protein was used as cut-off point to classify tumors as ER and/or PgR-positive. The following criteria were applied to include tumor specimens from final analysis: [1] sufficient frozen tumor material, [2] more than 30% epithelial tumor cells in hematoxylin and eosin stained sections, [3] breast tumor tissue specimen of good RNA quality according to predefined criteria (16), and [4] EZH2 mRNA expression levels were measured and reference mRNA levels were detectable. After applying the inclusion criteria, tumor specimens and clinical data of 1,318 patients were available for analysis. From these 1,318 patients (for clinicopathologic details, see Supplementary Table S1), 580 patients (44%) underwent breast conserving lumpectomy and 738 patients modified mastectomy (56%). The median follow-up time of patients alive was 90 months, range 4–231 months. Eight hundred and eighty-nine patients did not receive adjuvant systemic therapy, while 429 patients (33%; all lymph node-positive) did; 198 (15%) were treated with hormonal therapy, 216 (16%) with chemotherapy (70 patients anthracycline-based (FAC/FEC) and 146 patients non-anthracycline-based (CMF)) and 15 patients received both hormonal and chemotherapy.

HORMONAL THERAPY OF ADVANCED DISEASE

ER-positive tumors of 249 patients (out of the 1,318 M0 patients) who developed advanced disease after treatment for primary breast cancer and who received first-line tamoxifen therapy were included in this study. This set was completed with 29 tumors of patients with distant metastases at initial diagnosis (M1 patients). These 278 patients were divided according to response to tamoxifen. Clinical benefit from tamoxifen, defined as a complete or partial response according to standard International Union Against Cancer (Geneva, Switzerland) classification criteria (14) or no change longer than 6 months after treatment initiation (stable disease), was observed in 173 patients (62%); 11 patients showed complete response, 38 a partial response and 124 patients had stable disease. No clinical benefit occurred in 105 patients (38%).

RNA ISOLATION AND QUANTITATIVE REAL-TIME PCR

Tissue processing, RNA isolation, cDNA synthesis, quantitative real-time polymerase chain reaction (qRT-PCR) and expression data generation were performed as described previously (16). The qRT-PCR assays were carried out on an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) or a MX3000P Real-time PCR system (Stratagene, Amsterdam, The Netherlands). Assay-on-Demand kits (Applied Biosystems) were used to measure mRNA levels of EZH2 (Hs00544830_m1) and ER (Hs00174860_m1; measures ESR1). Primer sequences of the reference genes PGBD, HPRT, B2M have been described (16). Forty rounds of amplification were performed and fluorescent signals of Taqman probes were used to generate Cycle threshold (Ct) values to calculate mRNA expression levels. Expression levels of EZH2 and ER were normalized against average expression levels of three reference genes in tumor samples and against HPRT levels in cell lines (16).

BREAST CANCER CELL LINE AND RNA INTERFERENCE

MCF7, an estrogen sensitive ER-positive breast cancer cell line, was cultured in RPMI 1640 containing phenol red and 10% heat-inactivated fetal calf serum (Sigma–Aldrich Chemie). To perform EZH2 knockdown experiments, small interfering RNA (siRNA) targeting EZH2 mRNA (Qiagen, Venlo, The Netherlands) was used according recommendations and described previously (17). Two different siRNA duplexes for EZH2 were pooled with target sequences: r(CCAUGUUUACAACUAUCAA)dTdT (sense) and r(UUGAUAGUUGUAAACAUGG)dTdT (antisense) for the first duplex and r(GCAAAUUCUCGGUGUCAAA)dTdT (sense) and r(UUUGACACCGAGAAUUUGC)dTdT (antisense) for the second duplex. As control, MCF7 cells were transfected with non-specific silencing pool of siRNAs (Qiagen). siRNAs (5 nM) were introduced via inverse transfection into MCF7, using HiPerfect transfection reagent (Qiagen). Six experiments were independently performed at different time points in 24 (N = 1) or 96 wells plates (N = 5). The 24 and 96 wells plate contained 330,000 cells and 6,000 cells per well, respectively, and two and eight wells per condition was used and pooled for further analyses. Within three experiments in 96 wells plates, part of the cells was harvested after 96 h for mRNA and protein analysis. The remaining part was transfected with siRNAs again, and subsequently grown for 96 h in standard culture medium supplemented with the pure ER antagonist ICI 164384 (100 nM) or with ethanol vehicle alone as control. The pure ER antagonist ICI was used to exclude the agonistic effects of tamoxifen. To assess the effect of EZH2 silencing, cell numbers were counted with a coulter counter at day 4 and 8. To determine response to 96 h of ICI 164384 treatment cell numbers were counted at day 8. Throughout an experiment, culture medium was renewed every 3 days and at the end cells were lysed and RNA and protein isolated using the MirVana Paris kit (Ambion, Foster City, CA, USA).

IMMUNOCYTOCHEMISTRY AND WESTERN BLOTTING

Cytospins were prepared from MCF7 cells of above experiments, fixed with 1% formaldehyde and incubated with a monoclonal antibody against EZH2 (BD Biosciences, San Jose, CA, (1:2000)) and a secondary peroxidase conjugated rabbit anti-mouse antibody. EZH2 protein expression was visualized with a diaminobenzidine staining reaction. Western blotting of protein samples were performed as described previously (17). Antibodies against EZH2 (monoclonal (1:2000), BD Biosciences, 1:2000), H3K27 (polyclonal (1:5000), ABCAM, Cambridge, MA, USA) and GAPDH (monoclonal (1:500), Chemicon Inc., Temecula, CA, USA) were used and detected with horseradish peroxidase (HRP)-conjugated or HRP-polymer (DAKO Real Envision, DAKO, Diagnostica GmbH, Hamburg, Germany) labeled secondary antibodies and chemiluminescent reagents (ECL-kit, Pierce, Rockford, IL). The Scanalytics One-D program (Alpha Innotech Ltd., Cannock, UK) was used for quantification.

DATA ANALYSIS AND STATISTICS

The relationship of EZH2 expression levels with patient and tumor characteristics was investigated using non-parametric methods, i.e., Spearman rank correlations for continuous variables and Wilcoxon rank-sum or Kruskal–Wallis test for ordered variables. For the analyses with continuous variables, mRNA levels of EZH2 were log transformed and of ER and PgR were box-cox transformed to reduce skewness of the distribution. Logistic regression analysis was used to calculate the odds ratio (OR) that defines the relation between expression levels and clinical benefit from therapy. The Cox proportional hazards model was used to compute the hazard ratio (HR) in the analysis of metastasis-free survival (MFS), overall survival (OS) and progression-free survival (PFS). MFS and OS were previously described (18). PFS was defined as the time elapsed between initiation of tamoxifen therapy and the first detection of disease progression. In multivariate analysis, logistic and Cox regression analysis was applied to determine whether EZH2 had predictive value and was independent when added to the base model of traditional factors. The Cox proportional hazard assumption was not violated as verified by a test based on Schoenfeld residuals. Both HR and OR were represented with their 95% confidence intervals (95% CI). Survival curves were generated using the Kaplan and Meier method and a log rank test was used to test for differences. Computations were done with the STATA statistical package, release 10.1 (STATA Corp., College Station, TX). In the in vitro studies, a student *t*-test was performed to test for significance for differences in cell counts and RNA levels. All *P*-values were two-sided and $P < 0.05$ was considered statistically significant.

RESULTS

ASSOCIATIONS OF EZH2 MRNA EXPRESSION LEVELS WITH CLINICOPATHOLOGICAL FACTORS

In this study, we determined the mRNA expression levels in 1,318 primary breast carcinomas. Median expression levels of EZH2, its interquartile range, and its association with patient and tumor characteristics are summarized in Supplementary Table 1. Briefly, high EZH2 mRNA levels were significantly associated with younger age, premenopausal status, poor histologic grade, larger tumor size, and status of ER, PgR, ERBB2, and EGFR. An inverse correlation between EZH2 and ER was observed ($P < 0.001$, $r_s = -0.33$). Expression of EZH2 was higher in ERBB2 positive samples compared with ERBB2 negative samples ($P < 0.001$).

EZH2 LEVELS AND CLINICAL OUTCOME

The prognostic value of EZH2 was assessed in 688 lymph node-negative (LNN) patients who did not receive any adjuvant systemic therapy. EZH2 levels, as continuous variable, were not significantly correlated with MFS (HR = 1.14, 95% CI: 0.98–1.32; $P = 0.10$). Considering OS, EZH2 was significantly associated in LNN patients (HR = 1.25, 95% CI: 1.07–1.46; $P = 0.006$).

ASSOCIATION OF EZH2 LEVELS WITH CLINICAL BENEFIT AND PFS

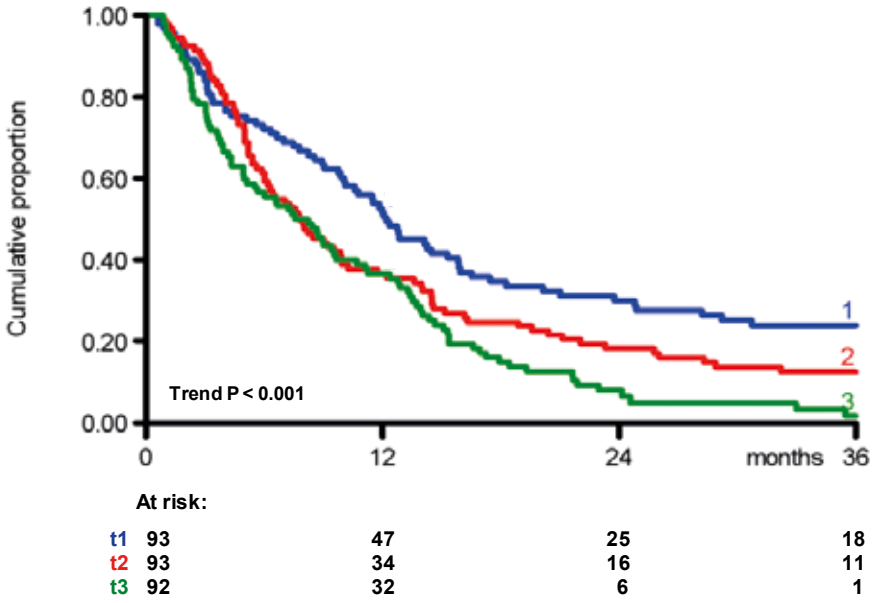
In univariate analysis, increasing EZH2 expression levels as continuous variable were significantly associated with a lower clinical benefit in patients with advanced breast cancer treated with first-line tamoxifen monotherapy ($N = 278$) (OR = 0.68, 95% CI: 0.47–0.98; $P = 0.04$) (Supplementary Table S2). In analogy, after categorizing EZH2 expression levels into tertiles, the highest tertile was significantly associated with a lower clinical benefit to tamoxifen therapy (OR = 0.48, 95% CI: 0.26–0.89; $P = 0.02$). In multivariate analysis, however, when corrected for traditional predictive factors including age, menopausal status, DSR, DFS, ER and PgR levels, no significant association with clinical benefit was observed (Supplementary Table S2). In univariate analysis, increasing EZH2 expression levels analyzed as continuous variable were significantly associated with shorter PFS (HR = 1.28, 95% CI: 1.08–1.51; $P = 0.004$) (Table 1). In univariate analysis, compared with tumors in the lowest tertile of EZH2 expression, those with the highest EZH2 levels were associated with a poor PFS (HR = 1.80, 95% CI: 1.32–2.46; $P < 0.001$) (Table 1; Figure 1). Remarkably, the intermediate and highest expression groups have a similar PFS during the first 12 months while the curves diverge thereafter. In multivariate analysis, compared with tumors with EZH2 levels in the lowest tertile, those with highest EZH2 levels were significantly associated with poor PFS (HR = 1.61, 95% CI: 1.16–2.24; $P = 0.004$). Moreover, ER levels were not significantly ($P = 0.21$) different between the 3 different tertiles of EZH2 (Supplemental Figure S1).

TABLE 1. Cox uni- and multivariate analysis for PFS of EZH2, continuous and categorized, in estrogen receptor-positive tumors from 278 patients whose recurrence was treated with first-line tamoxifen monotherapy.

Factor of base model		**Univariate analysis**			**Multivariate analysis***		
	N	**HR**	**95% CI**	***P***	**HR**	**95% CI**	***P***
Age (y)							
≤ 55	104 (37.4%)	1.00			1.00		
56-70	102 (36.7%)	0.83	0.62-1.10	0.19	0.68	0.45-1.03	0.07
>70	72 (25.9%)	0.68	0.50-0.94	0.02	0.60	0.38-0.93	0.02
Menopausal status							
Premenopausal	68 (24.5%)	1.00					
Postmenopausal	210 (75.5%)	0.90	0.67-1.19	0.45			
Disease-free survival							
≤ 1 year	72 (25.9%)	1.00			1.00		
1-3 years	124 (44.6%)	0.69	0.51-0.92	0.01	0.67	0.49-0.90	0.009
>3 years	82 (29.5%)	0.51	0.36-0.71	<0.001	0.50	0.36-0.71	<0.001
Dominant site of relapse							
Soft tissue	29 (10.4%)	1.00			1.00		
Bone	145 (52.2%)	1.29	0.85-1.96	0.23	1.29	0.81-2.01	0.26
Viscera	104 (37.4%)	1.18	0.76-1.83	0.45	1.39	0.87-2.21	0.17
ER mRNA	278 (100%)	0.90	0.86-0.96	<0.001	0.91	0.86-0.97	0.004
PgR mRNA	278 (100%)	0.90	0.85-0.96	0.001	0.92	0.86-0.98	0.01
Factors analyzed					**Additions to the base model**		
EZH2 mRNA							
Continuous variable	278 (100%)	1.28	1.08-1.51	0.004	1.26	1.05-1.52	0.015
Categorized variable							
t1	93 (33.5%)	1.00			1.00		
t2	93 (33.5%)	1.40	1.04-1.89	0.03	1.48	1.08-2.02	0.02
t3	92 (33.0%)	1.80	1.32-2.46	<0.001	1.61	1.16-2.24	0.004

* Stratified for postmenopausal status

FIGURE 1. Kaplan-Meier curves of PFS as a function of EZH2 mRNA expression levels. Patients were evenly divided into three groups according to EZH2 mRNA levels. Patients at risk at different time points are indicated.



EZH2 AND RNA INTERFERENCE

To investigate how EZH2 is functionally involved in response to anti-estrogens, we performed in vitro studies in the ER-positive human breast cancer cell line MCF7. To this end MCF7 cells were treated with ICI 164384 combined with non-silencing (NS) or EZH2 silencing. When cells transfected with siNS were exposed to 100 nM ICI 164384 a decrease in cell numbers of 25% (range 12–30%, $N = 3$, $P < 0.001$) after 96 h was observed, confirming that MCF7 is an anti-estrogen sensitive cell line. Knockdown experiments showed an average silencing level of EZH2 of 79% (range 64–93%, $N = 6$, $P < 0.001$) after 96 h (Figure 2). EZH2 silencing caused a significant decrease in cell numbers of 38% (range 17–53%, $N = 3$, $P < 0.001$) compared with controls (Figure 3). When EZH2 silencing and ICI-treatment were combined, cell numbers were reduced with 67% (range 54–75%, $N = 3$, $P < 0.001$) compared to non-silenced MCF7 cells. Both immunocytochemistry of cytospins and western blotting confirm knockdown of EZH2 on the protein level after 96 h of EZH2 silencing (Figure 4). To validate EZH2 silencing functionally we demonstrated (Figure 4) that methylation of lysine residue 27 of histone 3 diminishes when silencing EZH2.

FIGURE 2. EZH2 and ER mRNA expression levels after EZH2 silencing (siEZH2) in MCF7 cells. Average level of siEZH2 was 79% after 96 hours of culturing. Downregulation of EZH2 was associated with an average upregulation of ER of almost two-fold. Bars represent mean ± SD of triplicate measurements expressed as fold-difference compared with non-silenced MCF7 cells. Data shown are of six time-point independent cell culture experiments.

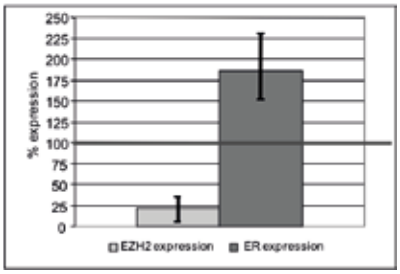


FIGURE 3. EZH2 silencing (siEZH2) in MCF7 and response to ICI 164384 treatment. MCF7 cells were cultured for 8 days in RPMI containing 10% FCS and transfected with siRNAs at day 0 and again at day 4. Culture medium was supplemented with ICI 164384 after 96 hours. Cell numbers were measured in siEZH2 and/or ICI 164384 at day 8. Bars represent mean ± SD of triplicate measurements expressed as fold-difference compared with untreated and non-silenced MCF7 cells. Results were obtained from 3 time-point independent cell culture experiments.

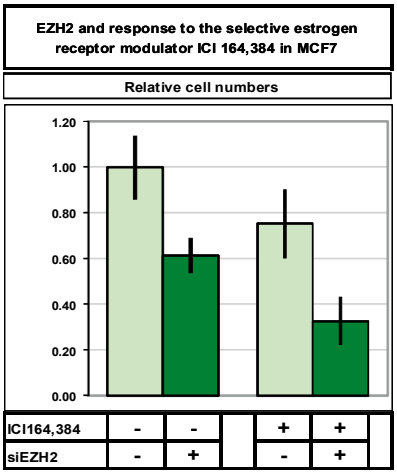
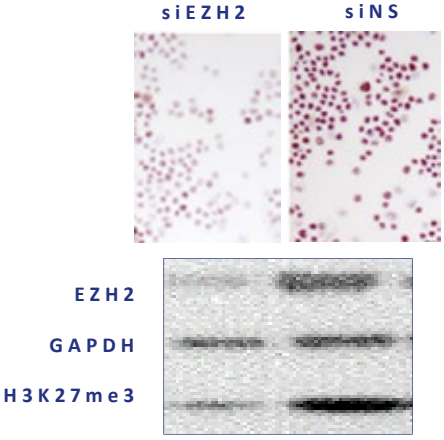


FIGURE 4. Immunocytochemistry of cytospins and western blotting. EZH2 protein expression after 96 h EZH2 silencing (siEZH2) compared with non-silenced MCF7 cells (siNS) is shown by immunocytochemistry (top). Western blotting (bottom) shows histone 3 lysine 27 trimethylation (H3K27me3).



ASSOCIATION BETWEEN EZH2 LEVELS AND ER EXPRESSION

In view of the inverse correlation between EZH2 and ER mRNA expression levels observed in our 1,318 breast cancer tumor specimens, we next studied the effect of EZH2 silencing on ER expression in vitro. Downregulation of EZH2 in MCF7 cells (79% after 96 h, range 64–93%, *N* = 6) was associated with an average two-fold upregulation of ER (*N* = 6, range 1.5–2.3, *P* = 0.001) (Figure 2), which is concordant with the observed inverse correlation between EZH2 and ER mRNA expression in clinical samples.

DISCUSSION

We demonstrate here for the first time that a significant association exists between high levels of EZH2 with outcome in terms of PFS for ER-positive breast cancer patients treated with first-line tamoxifen for advanced disease. Additionally, it was shown that EZH2 expression impacts response to tamoxifen rather than reflecting tumor aggressiveness since no prognostic value for EZH2 was revealed in LNN primary breast cancer who did not receive adjuvant systemic therapy.

In contrast to our findings, EZH2 has previously been suggested to bear prognostic value in breast cancer. Based on in silico analysis, on a relatively small set of 78 tumors of young LNN patients (<55 years) with low grade tumors (19), Kleer et al. reported that mRNA expression of EZH2 was significantly higher in invasive carcinomas that metastasize within 5 years of primary diagnosis compared with invasive carcinomas that did not (11). In another study immunohistochemistry was used to demonstrate an association between EZH2 expression and increased tumor cell proliferation in melanoma, prostate, endometrial, and breast cancer (9). Association of high EZH2 expression with unfavorable prognosis was revealed in all investigated tumor types with the exception of breast cancer as survival data were not available. We observed that EZH2 associates significantly with OS; however, OS is not only dependent on tumor aggressiveness but also on treatment and response of the patient in the adjuvant and/or advanced disease setting. That EZH2 in our study predicts poor OS in LNN patients who did not obtain adjuvant systemic therapy suggests an association with treatment response in the advanced disease setting, as for example shown for first-line tamoxifen monotherapy in this study. Collett et al. also used immunohistochemistry to assess the prognostic value of EZH2 in breast cancer (8). In 190 tumors (100 EZH2-negative and 90 EZH2-positive), they demonstrated a significant correlation between EZH2 positivity and high disease stage at time of diagnosis in terms of locally advanced disease or metastatic disease. However, this group comprises only six patients with metastatic disease at initial diagnosis. Additionally, this set was heterogeneous with both LNN and LN-positive tumors. And though treatment status was not discussed, it is reasonable to assume that LNP patients have received systemic treatment thereby impacting outcome and obscuring EZH2's true prognostic value. However, in our study population comprising the largest series of patients studied so far, 688 LNN patients, who did not receive any adjuvant systemic therapy enabling to assess the true prognostic value of

EZH2, we could not confirm its prognostic value. This discrepancy in findings remains to be clarified but might be due to the methodology applied and the cohorts of patients studied.

Furthermore, we found an association between EZH2 and the type of response to therapy in advanced disease with the highest EZH2 levels related to poor outcome. In order to elucidate the potential underlying mechanisms for this association, we explored the association between EZH2 expression and ER expression. In our 1,318 breast cancer tumor specimens, we observed an inverse association between EZH2 and ER mRNA expression levels. Accordingly, in silico analysis of 15 independent breast cancer datasets of the Oncomine database (Supplementary Figure S2), with in total 2,437 samples (713 ER-negative, 1724 ER-positive), confirmed this finding. In addition, we performed functional studies in which EZH2 expression was silenced with siRNAs in the human estrogen sensitive breast cancer cell line MCF7. The decrease in cell numbers following downregulating EZH2 expression in MCF7 suggests that EZH2 has an effect on cell proliferation, in agreement with previously performed studies showing an association between EZH2 and cell proliferation (8, 11). We have shown that growth inhibition in the experiment with ICI + siEZH2 (67% inhibition) adds up the effect of EZH2 silencing and ICI 164384 treatment on MCF7 growth (38 and 25% inhibition, respectively). These combined ICI + siEZH2 experiments demonstrate no overlap in growth inhibition and indicates that the effect of EZH2 silencing on MCF7 growth is independent of the effect of ER inhibition by ICI. Our observed upregulation of ER by silencing of EZH2 and the inverse correlation between EZH2 and ER status (ER-negative versus ER-positive) in breast cancer specimens (Supplemental Figure S2), however, suggest an EZH2 and ER interaction which may also result in enhanced sensitivity to ICI 164384. Although the observed two-fold upregulation of ER does not seem impressive at first glance, it is already observed after 96 h of culturing. Moreover, MCF7 is a cell line with already one of the highest ER expression levels in our panel of 39 breast cancer cell lines (data not shown). In that perspective, an almost two-fold upregulation can be regarded substantial. Furthermore, the extent of ER upregulation by silencing EZH2 is in the range of what has been described for other downstream factors. For example, Yu et al. published a 1.6-fold upregulation of the Adrenergic Receptor when silencing EZH2 (20). We hypothesize that the promoter region of ER lacks DNA methylation in MCF7 and that the observed upregulation of ER is predominantly due to decreased histone H3K27 trimethylation caused by EZH2 knockdown. Further studies are needed to verify this hypothesis.

Given the presumed role of EZH2 in the regulation of ER expression, EZH2 might be an interesting target for therapy. Recently, Varambally et al. discovered a physiological EZH2-inhibitor, miRNA-101, which inhibits the expression and function of EZH2 in cancer cell lines (21). It has been shown that miRNA-101 expression diminishes during cancer progression, resulting in an increased EZH2 expression and concomitant dysregulation of epigenetic pathways. This cascade is thought to underlie progression of several types of cancers, e.g., prostate, brain, and lung cancer (21). In addition, also miRNA-

26a has been reported to post-transcriptionally repress EZH2 (22). Recently, Kota et al. demonstrated the capacity of miRNA-26a as an anti-tumor therapy in a mouse model of hepatocellular carcinomas, where it resulted in inhibition of cancer cell proliferation and induction of tumor-specific apoptosis (23). As a result, it would be interesting to further investigate EZH2 as a potential target for therapy, and include miRNA-101 and miRNA-26a as “in vivo” inhibitors.

In conclusion, EZH2 has no prognostic value in our large set of LNN adjuvant untreated breast cancer patients. However, high EZH2 levels are associated with unfavorable outcome to tamoxifen treatment in breast cancer patients with advanced disease, which suggests that EZH2 can be used as a predictive marker. Moreover, downregulation of EZH2 caused additional growth inhibition next to anti-estrogen therapy in vitro and resulted in ER upregulation. If validated, EZH2 may be considered to serve as a potential target to increase the anti-tumor activity of anti-estrogen therapies in breast cancer. Furthermore, its assessment may contribute to a more appropriate selection of patients for tamoxifen therapy and thereby a more tailored management of patients with breast cancer.

REFERENCES

1. Jansen MP, Foekens JA, van Staveren IL, Dirkzwager-Kiel MM, Ritstier K, Look MP, et al. Molecular classification of tamoxifen-resistant breast carcinomas by gene expression profiling. J Clin Oncol. 2005;23(4):732-40.
2. Schuettengruber B, Chourrout D, Vervoort M, Leblanc B, Cavalli G. Genome regulation by polycomb and trithorax proteins. Cell. 2007;128(4):735-45.
3. Cao R, Wang L, Wang H, Xia L, Erdjument-Bromage H, Tempst P, et al. Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science. 2002;298(5595):1039-43.
4. Czermin B, Melfi R, McCabe D, Seitz V, Imhof A, Pirrotta V. Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. Cell. 2002;111(2):185-96.
5. Kuzmichev A, Nishioka K, Erdjument-Bromage H, Tempst P, Reinberg D. Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein. Genes & development. 2002;16(22):2893-905.
6. Margueron R, Li G, Sarma K, Blais A, Zavadil J, Woodcock CL, et al. Ezh1 and Ezh2 maintain repressive chromatin through different mechanisms. Molecular cell. 2008;32(4):503-18.
7. Jansen M, Foekens J, Ritstier K, Van Staveren I, Meijer-van Gelder M, Sieuwerts A, et al. A miniPathway for tamoxifen therapy resistance. Breast cancer research and treatment. 2005;94:S31-S.
8. Collett K, Eide GE, Arnes J, Stefansson IM, Eide J, Braaten A, et al. Expression of enhancer of zeste homologue 2 is significantly associated with increased tumor cell proliferation and is a marker of aggressive breast cancer. Clin Cancer Res. 2006;12(4):1168-74.
9. Bachmann IM, Halvorsen OJ, Collett K, Stefansson IM, Straume O, Haukaas SA, et al. EZH2 expression is associated with high proliferation rate and aggressive tumor subgroups in cutaneous melanoma and cancers of the endometrium, prostate, and breast. J Clin Oncol. 2006;24(2):268-73.
10. Raman JD, Mongan NP, Tickoo SK, Boorjian SA, Scherr DS, Gudas LJ. Increased expression of the polycomb group gene, EZH2, in transitional cell carcinoma of the bladder. Clin Cancer Res. 2005;11(24 Pt 1):8570-6.
11. Kleer CG, Cao Q, Varambally S, Shen R, Ota I, Tomlins SA, et al. EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells. Proceedings of the National Academy of Sciences of the United States of America. 2003;100(20):11606-11.
12. Varambally S, Dhanasekaran SM, Zhou M, Barrette TR, Kumar-Sinha C, Sanda MG, et al. The polycomb group protein EZH2 is involved in progression of prostate cancer. Nature. 2002;419(6907):624-9.
13. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM. REporting recommendations for tumor MARKer prognostic studies (REMARK). Breast cancer research and treatment. 2006;100(2):229-35.

14. Hayward JL, Carbone PP, Heuson JC, Kumaoka S, Segaloff A, Rubens RD. Assessment of response to therapy in advanced breast cancer: a project of the Programme on Clinical Oncology of the International Union Against Cancer, Geneva, Switzerland. Cancer. 1977;39(3):1289-94.
15. Foekens JA, Portengen H, van Putten WL, Peters HA, Krijnen HL, Alexieva-Figusch J, et al. Prognostic value of estrogen and progesterone receptors measured by enzyme immunoassays in human breast tumor cytosols. Cancer research. 1989;49(21):5823-8.
16. Sieuwerts AM, Meijer-van Gelder ME, Timmermans M, Trapman AM, Garcia RR, Arnold M, et al. How ADAM-9 and ADAM-11 differentially from estrogen receptor predict response to tamoxifen treatment in patients with recurrent breast cancer: a retrospective study. Clin Cancer Res. 2005;11(20):7311-21.
17. Jansen MP, Ruigrok-Ritstier K, Dorssers LC, van Staveren IL, Look MP, Meijer-van Gelder ME, et al. Downregulation of SIAH2, an ubiquitin E3 ligase, is associated with resistance to endocrine therapy in breast cancer. Breast cancer research and treatment. 2009;116(2):263-71.
18. van Agthoven T, Sieuwerts AM, Veldscholte J, Meijer-van Gelder ME, Smid M, Brinkman A, et al. CITED2 and NCOR2 in anti-oestrogen resistance and progression of breast cancer. British journal of cancer. 2009;101(11):1824-32.
19. van 't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, et al. Gene expression profiling predicts clinical outcome of breast cancer. Nature. 2002;415(6871):530-6.
20. Yu J, Cao Q, Mehra R, Laxman B, Yu J, Tomlins SA, et al. Integrative genomics analysis reveals silencing of beta-adrenergic signaling by polycomb in prostate cancer. Cancer cell. 2007;12(5):419-31.
21. Varambally S, Cao Q, Mani RS, Shankar S, Wang X, Ateeq B, et al. Genomic loss of microRNA-101 leads to overexpression of histone methyltransferase EZH2 in cancer. Science (New York, NY. 2008;322(5908):1695-9.
22. Wong CF, Tellam RL. MicroRNA-26a targets the histone methyltransferase Enhancer of Zeste homolog 2 during myogenesis. The Journal of biological chemistry. 2008;283(15):9836-43.
23. Kota J, Chivukula RR, O'Donnell KA, Wentzel EA, Montgomery CL, Hwang HW, et al. Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. Cell. 2009;137(6):1005-17.

SUPPLEMENTARY MATERIAL

https://drive.google.com/open?id=0B_eW3FoE0HzYdEJNZHlUcWx6ODg

CHAPTER 3

High miR-26a and low CDC2 levels associate with decreased EZH2 expression and with favorable outcome on tamoxifen in metastatic breast cancer

E.A. Reijm*, M.P.H.M. Jansen*, A.M. Sieuwerts, K. Ruigrok-Ritstier, M.P. Look,F.G. Rodríguez-González, A.A.J. Heine, J.W. Martens, S. Sleijfer, J.A. Foekens, E.M.J.J. Berns

Breast Cancer Res Treat 2012; 133(3): 937-947

* These authors contributed equally.

ABSTRACT

For patients with metastatic breast cancer, we previously described that increased EZH2 expression levels were associated with an adverse outcome to tamoxifen therapy. Main objective of the present study is to investigate miR-26a and miR-101 levels, which both target EZH2, for their association with molecular pathways and with efficacy of tamoxifen as first-line monotherapy for metastatic breast cancer. Expression levels were measured using quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) in primary breast cancer specimens of 235 estrogen receptor-α (ER)-positive patients. Pathway analysis was performed on microarray data available for 65 of these tumors. Logistic regression and Cox uni- and multivariate analysis were performed to relate expression levels with clinical benefit and time to progression (TTP). Increasing levels of miR-26a were significantly ($P < 0.005$) associated with both clinical benefit and prolonged TTP, whereas miR-101 was not. Cell cycle regulation and CCNE1 and CDC2 were the only significant overlapping pathway and genes differentially expressed between tumors with high and low levels of miR-26a and EZH2, respectively. In addition, increasing mRNA levels of CCNE1 ($P < 0.05$) and CDC2 ($P < 0.001$) were related to poor outcome. Multivariate analysis revealed miR-26a and CDC2 as an optimal set of markers associated with outcome on tamoxifen therapy, independently of traditional predictive factors. To summarize, only miR-26a levels are related with treatment outcome. Cell cycle regulation is the only overlapping pathway linked to miR-26a and EZH2 levels. Low mRNA levels of EZH2, CCNE1, and CDC2, and high levels of miR-26a are associated with favorable outcome on tamoxifen.

INTRODUCTION

The anti-estrogen tamoxifen has been used for more than three decades for the treatment of estrogen receptor-α (ER)-positive breast cancer in both adjuvant and metastatic settings. The majority of breast tumors express ER, however, half of the patients with metastatic disease initially fail to respond to endocrine therapy, while the remaining patients will develop resistance during therapy. More insight into factors underlying tamoxifen resistance as well biomarkers to identify patients likely to benefit from tamoxifen is therefore needed.

We identified and validated an 81-gene signature that predicts tamoxifen resistance in patients with metastatic breast cancer (1, 2). This signature included a member of the Enhancer of Zeste Homolog (EZH) family, which consists of EZH1 (OMIM 601674) and EZH2 (OMIM 601573). EZH2 is one of the polycomb proteins, a highly conserved group of chromatin modifiers known for their role in epigenetic memory and preservation of cellular characteristics (3). Our in vitro studies showed that knockdown of EZH2 upregulates ER as a consequence of which sensitivity to anti-estrogen therapy increases (4). In line with this, we have validated the predictive value of EZH2 and showed that low EZH2 levels were associated with favorable outcome on tamoxifen treatment in breast cancer patients with metastatic disease (4).

MicroRNAs (miRs) consist of a family of endogenously expressed small noncoding RNAs that target coding mRNAs to repress translation or induce degradation of their target mRNAs (5). There is accumulating evidence that misregulation of miRs plays an important role in cancer. In breast cancer, miRs have been related with metastatic behavior, clinical outcome and ER status (6, 7). Expression of several miRs in ER-positive breast cancer have also been associated with response to tamoxifen in cell lines (miR-221 and -222) (8), and in patients with metastatic disease treated with first-line tamoxifen (miR-30a, -30c, and -182) (9).

With respect to EZH2, miR target prediction tools have indicated that several miRs can target EZH2, but only two miRs, i.e., miR-26a and miR-101, have actually been shown to regulate EZH2 expression in different tissues (10, 11). In the present study, we examined whether miR-26a and miR-101 were associated with EZH2 mRNA levels in breast cancer and with outcome on first-line tamoxifen therapy. In addition, using available whole genome mRNA data from a subset of tumors, the global testing approach (GTA) was performed to identify molecular pathways correlated with expression levels of miR-26a, miR-101, and EZH2 and to reveal genes, within these pathways, that associate with outcome on tamoxifen.

PATIENTS AND METHODS

PATIENTS

Frozen breast tumor tissue specimens from female patients with primary operable breast cancer, who entered the clinic between 1981 and 1996 were analyzed. Follow-up, tumor staging, and response to therapy were performed as defined by standard International Union Against Cancer (Geneva, Switzerland) classification criteria (12). This retrospective study was performed in accordance with the Code of Conduct of the Federation of Medical Scientific Societies in the Netherlands (http://www.fmwv.nl), and reported following the REMARK recommendations (13), wherever possible. The study has been approved by the medical ethics committee of the Erasmus MC Rotterdam, The Netherlands (MEC 02.953).

Tumor protein expression levels of ER and progesterone receptor (PgR) were determined and used to classify tumors as ER- and/or PgR-positive as described previously (4, 14). The following criteria were applied to include breast tumor specimens for final analysis in this study: [1] sufficient frozen tumor material, [2] more than 30% epithelial tumor cell nuclei in haematoxylin/eosin-stained sections, and [3] specimen of good RNA quality according to predefined criteria (15). After applying these criteria, 235 patients with ER-positive tumors, who had metastatic disease treated with tamoxifen as first-line therapy, were included in this study. From these 235 patients, 89 patients (38%) underwent breast-conserving lumpectomy and 146 patients modified mastectomy (62%). The median follow-up time of patients alive was 89 months, range 10–165 months. Hundred and sixty five patients (70%) did not receive prior adjuvant systemic therapy, while 42 patients (18%) were previously treated with adjuvant chemotherapy [25 patients (11%) with non-anthracycline-based (CMF) and 17 patients (7%) with anthracycline-based (FAC/FEC) regimens].

Twenty eight patients (12%) presented with distant metastases at initial diagnosis (M1 patients). Clinical benefit on first-line tamoxifen monotherapy, defined as a complete or partial response according to standard International Union Against Cancer (Geneva, Switzerland) classification criteria (12) or no change longer than 6 months after treatment initiation (stable disease), was observed in 148 patients (63%). Eleven patients (5%) showed a complete response, 33 (14%) a partial response, and 104 patients (44%) had stable disease. No clinical benefit occurred in 87 patients (37%). Time to progression (TTP) was defined as the time elapsed between initiation of tamoxifen therapy and first detection of disease progression.

TABLE 1. Associations of the expression levels for miR-26a, miR-101, CDC2, and CCNE1 with patient and tumor characteristics.

Clinicopathological factors	*N*	%	miR-26a			miR-101			*N*	%	CCNE1			*N*	%	CDC2		
			Median	iqr[a]	*P**	Median	iqr[a]	*P**			Median	iqr[a]	*P**			Median	iqr[a]	*P**
Total	235 (65)	100 (28)	0.99	0.41		1.03	0.81		226	100	0.03	0.03		230	100	9.94	7.11	
Age in categories, year					0.21[b]			0.09[b]					0.60[b]					0.40[b]
<40	12 (2)	5 (3)	1.04	0.66		1.19	0.90		12	5	0.03	0.03		12	5	9.41	0.90	
41–55	75 (22)	32 (34)	0.95	0.38		0.93	0.55		73	32	0.03	0.03		75	33	11.09	0.56	
56–70	89 (26)	38 (40)	1.01	0.38		1.04	0.87		84	37	0.03	0.03		89	39	9.42	0.87	
>70	59 (15)	25 (23)	1.05	0.45		1.25	0.78		57	25	0.03	0.03		54	23	10.05	0.78	
Menopausal status					0.07[c]			0.036[c]					0.07[c]					0.14[c]
Premenopausal	56 (17)	24 (26)	0.95	0.40		0.95	0.66		55	24	0.03	0.03		56	24	11.14	6.31	
Postmenopausal	179 (48)	76 (74)	1.02	0.43		1.07	0.83		171	76	0.03	0.03		174	76	9.85	7.36	
Tumor size					0.74[d]			0.12[d]					0.45[d]					0.16[d]
pT1, <2 cm	63 (32)	27 (49)	0.99	0.46		0.90	0.54		60	27	0.03	0.03		62	27	10.00	7.46	
pT2, >2–5 cm	140 (30)	60 (46)	0.97	0.39		1.10	0.84		136	60	0.03	0.03		137	60	9.81	7.14	
pT3, >5 cm + pT4	32 (3)	14 (5)	1.05	0.49		1.07	1.04		30	13	0.03	0.03		31	13	11.42	10.11	
Lymph nodes involved[f]					0.79[d]			0.61[d]					0.61[d]					0.76[d]
0	96 (64)	44 (98)	0.98	0.37		0.99	0.57		92	41	0.03	0.03		96	42	9.96	7.62	
1–3	55 (1)	25 (2)	1.02	0.45		1.03	0.87		52	23	0.03	0.03		54	23	9.97	6.57	
>3	69 (0)	31 (0)	0.96	0.47		1.08	0.87		67	30	0.03	0.03		68	30	9.93	9.36	
Grade					0.15[d]			0.41[d]					0.13[d]					0.41[d]
Poor	134 (33)	57 (51)	0.93	0.44		1.05	0.80		126	56	0.03	0.03		131	57	10.31	7.01	
Unknown	72 (24)	31 (37)	1.05	0.37		0.99	0.74		71	31	0.02	0.02		71	31	9.55	5.64	
Good/moderate	29 (8)	12 (12)	0.97	0.44		0.99	0.83		29	13	0.04	0.04		28	12	9.35	11.54	
PgR status[e,f]					<0.001[b]			<0.001[b]					<0.001[b]					<0.001[b]
PgR low	44 (16)	19 (25)	0.87	0.31		0.76	0.63		43	19	0.03	0.04		43	19	12.84	9.23	
PgR high	185 (47)	79 (75)	1.03	0.42		1.07	0.78		177	78	0.03	0.03		181	79	9.55	6.98	

TABLE 1. (continued)

Clinicopathological factors	*N*	%	miR-26a			miR-101			*N*	%	CCNE1			*N*	%	CDC2		
			Median	iqr[a]	*P**	Median	iqr[a]	*P**			Median	iqr[a]	*P**			Median	iqr[a]	*P**
HER2 status[c,e,f]					0.026[c]			0.061[c]					0.003[c]					0.026[c]
HER2 low	197 (51)	84 (84)	1.02	0.41		1.03	0.83		189	84	0.03	0.03		192	83	9.75	7.11	
HER2 high	34 (10)	14 (16)	0.88	0.37		0.81	0.70		33	15	0.05	0.03		34	15	11.63	8.22	
EGFR levels[e,f]					0.044[b]			0.081[b]					<0.001[b]					0.50[b]
EGFR low	118 (27)	50 (42)	1.02	0.44		1.07	0.83		114	50	0.03	0.03		115	50	10.06	7.72	
EGFR high	117 (38)	50 (58)	0.97	0.40		0.99	0.65		112	50	0.03	0.03		115	50	9.91	7.01	

The number between brackets in the columns presenting the number and percentage of patients indicate the patient frequency for the 65 samples evaluated in the GTA for pathways.

* Two-sided *P* value

[a] Interquarter range (q75-q25)

[b] Spearman rank correlation

[c] Mann–Whitney U test

[d] Kruskal–Wallis test

[e] Low and high seroid hormone receptor protein status as defined in the “Methods” section

[f] Nodal status, PR and HER2 status were not known or determined in 15, 6 and 4 samples, respectively

METHODS

Details of applied methodologies are available at Supplemental Methods. In brief, tissue processing, RNA isolation, cDNA synthesis, quantitative Real-Time Polymerase Chain Reaction (qRT-PCR), and expression data generation were performed as described previously (15). For pathway analysis, samples with whole genome mRNA expression profiles available, measured on Affymetrix HG-U133A and Plus2 chips, were selected (N = 65, 28%) and only reliable, i.e., quality checked, probes (N = 10,520) were evaluated. Samples were grouped according to median expression levels of miR-26a, miR-101 or EZH2. The Global Test Approach (GTA) was used to identify KEGG/BioCarta biological pathways in genes co-expressed with the biomarker of interest. Pathways were taken into account when P-values, after correction for multiple testing and resampling, were below 0.05 and genes with z-scores >1.96 were considered significant contributors to the pathways. The GTA package version 4.14.0 was run in the R version 2.9.0. Data analysis and statistics were performed as previously described (4). Expression levels of miR-26a, miR-101, and EZH2, CCNE1, CDC2, ER, and PgR mRNA levels were transformed to reduce distribution skewness. Logistic regression analysis was used to compute the odds ratio (OR) for clinical benefit and the Cox proportional hazards model to calculate the hazard ratio (HR) for TTP. Computations were done with the STATA statistical package, release 11.1 (STATA Corp., College Station, TX). All P-values were two-sided, and $P < 0.05$ was considered as statistically significant.

RESULTS

ASSOCIATIONS WITH CLINICOPATHOLOGICAL FACTORS

In this study, we determined the miR-26a, miR-101, and EZH2 mRNA expression levels in 235 primary breast carcinomas. The median and interquartile ranges of expression levels for miR-26a were 0.99 and 0.41, for miR-101 were 1.03 and 0.81 and for EZH2 were 0.10 and 0.07. The miR-26a and miR-101 levels correlated with each other (r_s = 0.43, $P < 0.001$) and showed an inverse relation with EZH2 mRNA levels (r_s = −0.21 and r_s = −0.15, respectively, $P < 0.05$). Expression levels of both miRs were not significantly related with age, tumor grade, tumor size, or nodal status (Table 1). Only miR-101 levels were associated with postmenopausal status (P = 0.036). The ER and PgR mRNA levels showed a significant positive correlation with those of miR-26a (r_s = 0.21 and r_s = 0.34, for both $P < 0.002$) and miR-101 (r_s = 0.13, P = 0.04 and r_s = 0.27, $P < 0.001$).

ASSOCIATIONS WITH CLINICAL BENEFIT AND TIME TO PROGRESSION

Expression levels of miR-26a, miR-101 and EZH2 mRNA levels were evaluated in uni- and multivariate analysis for their associations with clinical benefit (Supplemental Table 2) and TTP (Table 2) in patients with metastatic breast cancer treated with tamoxifen as first-line monotherapy. The miR-101 levels were not related with clinical benefit (OR = 0.84, *P* = 0.40) nor with TTP (Table 2). As continuous variable, increasing levels of miR-26a were significantly associated with clinical benefit (OR = 32.1, *P* < 0.001) and with favorable TTP (HR = 0.13, *P* < 0.001; Table 2). Increasing mRNA levels of EZH2 were related to lower chance of clinical benefit (OR = 0.61, *P* = 0.02) and shorter TTP (HR = 1.26, *P* = 0.02). Analysis of miR-26a and EZH2 categorized in thirds (i.e. three quantiles) showed that the third with highest levels of miR-26a was related to clinical benefit (OR = 4.10, *P* < 0.001) and with prolonged TTP (HR = 0.43, *P* < 0.001), whereas the third with the highest EZH2 levels correlated with treatment failure (OR = 0.34, *P* = 0.002) and shorter TTP (HR = 1.91, *P* < 0.001). Kaplan–Meier curves as function of categorized expression levels of miR-26a and EZH2 visualize their association with TTP (Figure 1). The median differences in TTP were 6.5 months between patients with high and low expression levels for miR-26a and 5.6 months for those with high and low EZH2 expression levels. In multivariate analysis, when added separately to the base model of predictive factors, miR-26a and EZH2 were significantly associated with clinical benefit and TTP, both as continuous and as categorized variables. Patients with high miR-26a levels showed clinical benefit (OR = 3.31, *P* = 0.005) and the longest TTP (HR = 0.52, *P* < 0.001), whereas those with high EZH2 levels had less benefit (OR = 0.39, *P* = 0.02) and shorter TTP (HR = 1.80; *P* = 0.001). The results of the multivariate analysis show the independence of miR-26a and EZH2 from traditional predictive factors included in the base model.

TABLE 2. Cox uni- and multivariate analysis for TTP in patients with metastatic disease treated with tamoxifen.

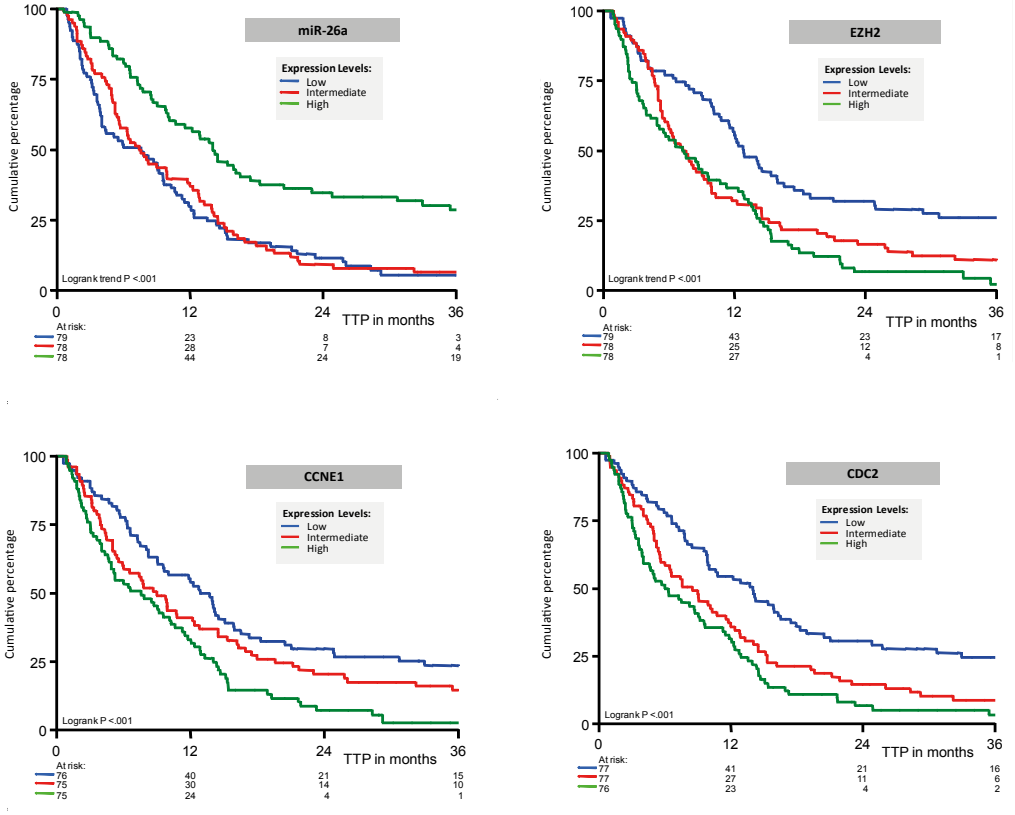
			Univariate analysis			Multivariate analysis*		
Factor of base model	***N***	**%**	**HR**	**95% CI**	***P***	**HR**	**95% CI**	***P***
Age (y)								
≤ 55	87	37	1.00			1.00		
55-70	89	38	0.82	0.60-1.11	0.19	0.71	0.45-1.11	0.13
>70	59	25	0.66	0.47-0.94	0.02	0.58	0.36-0.94	0.03
Menopausal status								
Premenopausal	56	24	1.00					
Postmenopausal	179	76	0.86	0.63-1.17	0.33			
Disease-free survival								
≤ 1 year	62	26	1.00			1.00		
1-3 years	109	46	0.66	0.48-0.91	0.01	0.63	0.46-0.88	0.006
>3 years	64	27	0.51	0.35-0.75	<0.001	0.52	0.36-0.77	0.001

TABLE 2. (continued)

			Univariate analysis			Multivariate analysis*		
Factor of base model	***N***	**%**	**HR**	**95% CI**	***P***	**HR**	**95% CI**	***P***
Dominant site of relapse								
Soft tissue	26	11	1.00			1.00		
Bone	127	54	1.29	0.83-2.02	0.26	1.28	0.79-2.07	0.31
Viscera	82	35	1.12	0.70-1.79	0.64	1.29	0.77-2.15	0.33
ER mRNA	235	100	0.89	0.83-0.94	<0.001	0.90	0.84-0.96	0.002
PgR mRNA	235	100	0.90	0.84-0.96	0.002	0.91	0.85-0.98	0.02
Factors analyzed							*Additions to base model*	
miR-26a								
Continuous variable	235	100	0.13	0.06-0.28	<0.001	0.18	0.07-0.44	<0.001
Low	79	34	1.00			1.00		
Intermediate	78	33	0.93	0.68-1.29	0.68	1.18	0.83-1.66	0.35
High	78	33	0.43	0.31-0.61	<0.001	0.52	0.36-0.76	<0.001
miR-101								
Continuous variable	235	100	0.87	0.70-1.07	0.19	0.90	0.71-1.13	0.37
EZH2 mRNA								
Continuous variable	235	100	1.26	1.06-1.51	0.01	1.28	1.05-1.56	0.02
Low	79	34	1.00			1.00		
Intermediate	78	33	1.58	1.14-2.19	0.006	1.73	1.23-2.44	0.002
High	78	33	1.91	1.37-2.68	<0.001	1.80	1.26-2.55	0.001
CCNE1 mRNA								
Continuous variable	226	96	1.27	1.12-1.45	<0.001	1.24	1.06-1.44	0.007
Low	76	34	1.00			1.00		
Intermediate	75	33	1.19	0.85-1.66	0.31	1.24	0.88-1.76	0.22
High	75	33	1.87	1.33-2.62	<0.001	1.62	1.11-2.35	0.01
CDC2 mRNA								
Continuous variable	230	98	1.53	1.29-1.81	<0.001	1.54	1.27-1.87	<0.001
Low	77	34	1.00			1.00		
Intermediate	77	33	1.53	1.09-2.13	0.01	1.52	1.07-2.15	0.02
High	76	33	2.07	1.47-2.90	<0.001	2.05	1.42-2.98	<0.001
miR-26a & CDC2								
miR-26a	230	98	0.22	0.09-0.52	<0.001	0.27	0.11-0.65	0.004
CDC2	230	98	1.38	1.15-1.65	0.001	1.47	1.20-1.79	<0.001

*The multivariate analysis is stratified for menopausal status

FIGURE 1. Kaplan-Meier curves of TTP as a function of miR-26a, EZH2, CCNE1 and CDC2 expression levels. Patients were evenly divided into three groups according to their expression levels. Curves were generated as function of low, intermediate, and high miR-26a, EZH2, CCNE1 and CDC2 expression levels. Patients at risk at different time points are indicated.



PATHWAY ANALYSIS FOR MIR-26A AND EZH2

In an exploratory pathway analysis with GTA, we evaluated 109 KEGG/BioCarta biological pathways and 10,520 mRNAs for differentially expressed pathways and genes. GTA identified only two pathways which significantly correlated with miR-26a, and 10 pathways with EZH2 mRNA expression (Table 3). The cyclins and cell cycle regulation pathway, and genes CCNE1 and CDC2 were the only overlapping pathway and genes between miR-26a and EZH2 that contributed significantly (Figure 2). Increased expressions of CCNE1 and CDC2 were observed in samples with low miR-26a levels and in samples with high EZH2 levels.

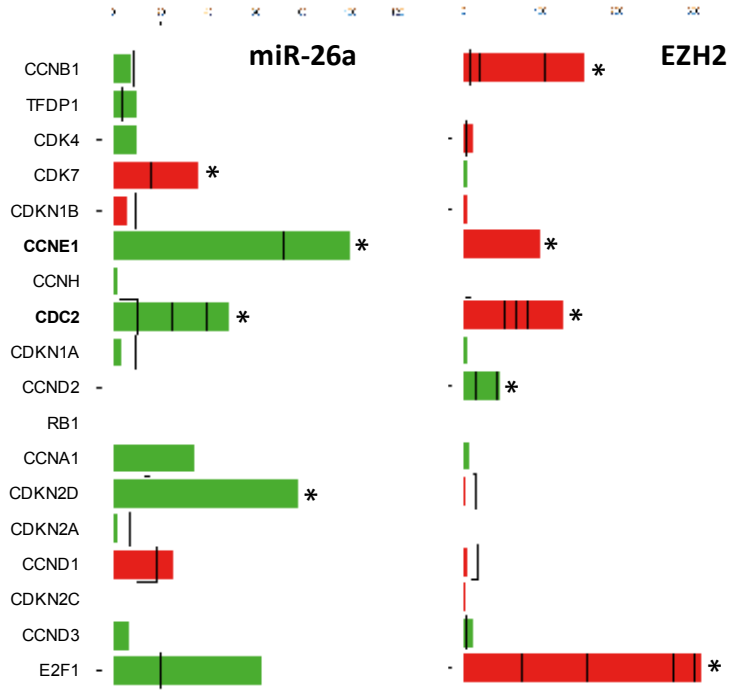
To confirm this exploratory analysis, the predictive value of CCNE1 and CDC2 was evaluated by qRT-PCR. The median and interquartile mRNA levels were 0.03 and 0.03 for CCNE1 (N = 226), and 9.94 and 7.11 for CDC2 (N = 230), respectively. The mRNA levels of CCNE1 and CDC2 correlated with each other (r_s = 0.44, $P < 0.001$) and showed a positive association with EZH2 mRNA levels (r_s = 0.45 and r_s = 0.57, for both $P < 0.001$) and an inverse relation with miR-26a (r_s = −0.44 and r_s = −0.30, respectively, for both $P \leq 0.001$). The ER and PgR mRNA expression levels showed an inverse correlation with those of CCNE1 (r_s = −0.14, P = 0.03 and r_s = −0.24, $P < 0.001$) and CDC2 (r_s = −0.07, P = 0.32 and r_s = −0.27, ($P < 0.001$). Expression levels of CDC2 and CCNE1 were not related with age, menopausal status, tumor grade, tumor size, or nodal status (Table 1). In univariate analysis, increasing mRNA levels of CCNE1 were related to treatment failure (OR = 0.67, P = 0.005; Supplemental Table 2) and shorter TTP (HR = 1.27, $P < 0.001$; Table 2). In addition, increased expression of CDC2 was associated with poor clinical benefit (OR = 0.45, $P < 0.001$) and TTP (HR = 1.53, $P < 0.001$). In multivariate analysis, CCNE1 and CDC2, when added separately to the base model, were both independent from traditional predictive factors for their association with clinical benefit and TTP (Supplemental Table 2; Table 2). Categorized into thirds, Kaplan–Meier survival analysis showed that patients with higher mRNA levels of CCNE1 and CDC2 had a shorter TTP (Figure 1). Compared to the group with low tumor levels of CCNE1, those with high levels of CCNE1 had an OR of 0.33 (P = 0.002) and a HR of 1.87 ($P < 0.001$), respectively. Patients with high tumor levels of CDC2 had an OR of 0.28 ($P < 0.001$) and even a HR of 2.07 ($P < 0.001$), respectively, compared with those with low tumor CDC2 levels. These results indicate that an activated cell cycle regulation pathway through increased expressions of CCNE1 and CDC2 is significantly associated with poor outcome on tamoxifen therapy. Moreover, two additional cyclins and cell cycle regulation pathway genes (E2F1 and CCNB1) were evaluated, next to CCNE2 (not in GTA because it failed quality control), to confirm the involvement of the cell cycle regulation pathway in the response to tamoxifen. All three genes showed a significant association with TTP in uni- and multivariate analyses as continuous variables, i.e., E2F1 had a HR of 1.38 (P = 0.013), CCNE2 had a HR of 1.38 ($P < 0.001$) and CCNB1 had a HR of 1.86 ($P < 0.001$) (Supplemental Table 3).

TABLE 3. miR-26a and EZH2 related pathways and genes.

Global Testing Approach - KEGG/BioCarta Pathway Analysis			
	Genes Tested	***P***	**Genes significant (z-score >1.96)**
miR-26a associated pathways			
Cyclins and Cell Cycle Regulation	18	**0.008**	**CCNE1**,CDK7,CDKN2D,**CDC2**
TPO Signaling Pathway	18	0.018	HRAS,THPO,RASA1
EZH2 associated pathways			
Cell Cycle G1 S Check Point	21	0.002	TGFB1,E2F1,ATM,SMAD4,CDC2, CCNE1,SKP2,ATR,ABL1
Role of BRCA1 BRCA2 and ATR in Cancer Susceptibility	20	0.003	FANCG,RAD51,ATM,FANCA,CHEK1, ATR,RAD9A,NBN,FANCC,BRCA1
Cyclins and Cell Cycle Regulation	18	**0.005**	CCNB1,E2F1,**CDC2**,**CCNE1**,CCND2
ATM Signaling Pathway	16	0.011	RAD51,ATM,NFKB1,CHEK1, GADD45A,ABL1,NBN,BRCA1
Spliceosomal Assembly	15	0.018	SNRPD1,SNRPG,SNRPF,U2AF1, SFRS2,U2AF2,SNRPE,SNRPA1
Cytokines and Inflammatory Response	15	0.019	TGFB1,HLA-DRA,IL15,CD4,CSF1,LTA
Cell Cycle G2 M Checkpoint	21	0.025	CCNB1,ATM,CDC2,PLK1,CHEK1, ATR,WEE1,GADD45A,BRCA1
ADPRibosylation Factor	15	0.029	KDELR1,ARFGAP1,DDEF2, PSCD4,COPA,CENTD1
Hypoxia and p53 in the Cardiovascular system	16	0.038	ATM,FHL2,CSNK1A1,GADD45A
p38 MAPK Signaling Pathway	32	0.044	TGFB1,CREB1,DAXX,CDC42,DDIT3, MAPKAPK5,HMGN1,HRAS,PLA2G4A

In 65 breast cancer samples, for which whole-genome mRNA expression profiles were available, pathways and genes were identified with the GTA of 109 KEGG/BioCarta biological pathways and 10,520 mRNAs. Only those pathways and their genes are indicated, which show a significant relationship with miR-26a and EZH2 expression levels. The number of genes tested is indicated per pathway. The *P*-values determine the significance of the association after correction for multiple testing and resampling.

FIGURE 2. Global testing approach (GTA) result of the cyclins and cell cycle regulation pathway. This pathway was overlapping between miR-26a- and EZH2-related pathways. *Red bars* illustrate high expression levels of the pathway gene in samples with high miR-26a or EZH2 levels, whereas *green bars* indicate high expression levels in samples with low miR-26a or EZH2 levels. The *number of vertical markers* in a *bar* indicates the significance and the height of a bar the contribution of a gene to the pathway. The *continuous line* shows the threshold for significance; bars with more than two *lines* above this border are significantly ($P < 0.05$) differentially expressed genes within the pathway, which are also indicated with an *asterisk*. Only CCNE1 and CDC2 showed significant associations with both miR-26a and EZH2.



MULTIVARIATE ANALYSIS OF MIR-26A, EZH2, CCNE1, AND CDC2

To determine a set of predictive biomarkers, the expression of miR-26a levels and of EZH2, CCNE1, and CDC2 mRNA levels were added simultaneously in a multivariate analysis to evaluate their relationship with TTP. Both CCNE1 and EZH2 mRNA levels lost their predictive value when included with miR-26a and CDC2, defining miR-26a and CDC2 levels as the set of predictive biomarkers associated with TTP. The HRs in the simultaneous analysis of miR-26a and CDC2 as continuous variables were 0.22 ($P < 0.001$) and 1.38 ($P = 0.001$), respectively (Table 2). Their contribution to the multivariate base model was independent from traditional predictive factors included in the model (Table 2). Converting miR-26a and CDC2 levels into a score followed by categorization into thirds resulted in a HR of 1.90 for the group with intermediate scores and a HR of 3.03 for the group with highest scores (see Supplemental Figure 1 for Kaplan–Meier survival curves).

DISCUSSION

This study shows that miR-26a levels associate with outcome of metastatic disease on first-line tamoxifen monotherapy, whereas miR-101 does not. Patients with clinical benefit have high miR-26a and low EZH2 mRNA levels. Additionally, only the cell cycle regulation pathway with its genes CCNE1 and CDC2 overlap between miR-26a and EZH2 linked molecular pathways. These two genes also correlate with treatment outcome. The miR-26a and CDC2 levels that regulate EZH2 levels and activity were identified as a set of predictive biomarkers for treatment outcome.

Overexpression of EZH2 was observed in prostate and breast cancer in which it was associated with aggressive clinical behaviour (16, 17). We demonstrated that decreased EZH2 mRNA levels were predictive for favorable outcome on tamoxifen in metastatic breast cancer (4). Both miR-26a and miR-101 repress EZH2 expression (10, 11, 18). Although miR-26a and miR-101 expressions correlate with EZH2 levels in our current study, only miR-26a had a significant association with outcome on tamoxifen. Expression of miR-26a is repressed by estrogens in vitro and is induced in breast cancer patients treated with anti-estrogen neoadjuvant therapy (19) whereas miR-101 expression is upregulated by androgen stimulation (18), but is not regulated by estrogens (19, 20). The fact that androgens stimulate miR-101 expression, whereas estrogens repress miR-26a expression needs to be elucidated, but suggests that EZH2 repression by miR-26a and miR-101 might be tissue as well as hormone dependent. That only miR-26a and not miR-101 has a relation with treatment outcome is because these miRs target many other genes. Of the genes predicted to be targets of miR-26a (1,012 targets) and miR-101 (1,198 targets), only a few (66 genes, including EZH2) are targeted by both miRs (data not shown). We cannot exclude another relevant gene for endocrine therapy outcome as specific miR-26a target which is not targeted by miR-101. This certainly needs further exploration but is not within the scope of the current study.

Our pathway analyses identified only the cell cycle regulation pathway to be correlated with miR-26a and EZH2 levels. The genes CDK7, CCNE1, CDC2, and CDKN2D for miR-26a and CCNB1, CCNE1, CDC2, CCND2, and E2F1 for EZH2 were differentially expressed within this pathway. CCNE2 and CDK2, important genes in this pathway, were not included in the analyses because their probes failed quality control. The association of EZH2 with cell cycle regulation is extensively reported (21, 22). Moreover, the Targetscan algorithm predicted cyclins D2, E1, and E2 (CCND2, CCNE1, and CCNE2), and cyclin dependent kinase 6 (CDK6), which all play a role in the G1–S transition, as miR-26a targets (23). Finally, estrogens that regulate G1 cyclin-dependent kinases (24) and tamoxifen has a cytostatic effect on breast cancer cells and arrest them in G0/G1 phase (25).

Based on our study, CCNE1 and CDC2 were the only overlapping genes for miR-26a and EZH2. We have shown earlier Cyclin E as prognostic marker for lymph node-negative breast cancer (26). Now, we show that in the metastatic disease setting, high CCNE1 mRNA levels correlate with poor outcome on tamoxifen. In concordance, patients

with high CCNE protein levels had less benefit from tamoxifen in an adjuvant setting (27), and the overexpression of low molecular weight CCNE isoforms was associated with resistance to fulvestrant (28) and letrozole (29). CCNE1 is a kinase and regulatory subunit of CDK2 that accumulates at the G1–S phase (30).

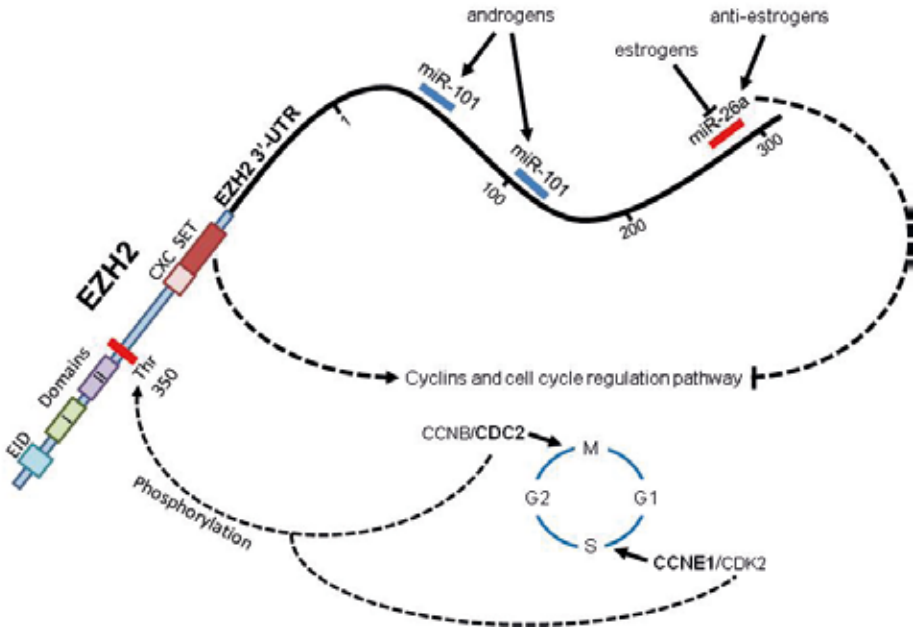
The second gene, CDC2 [also known as cyclin-dependent kinase 1 (CDK1)], correlated with miR-26a and EZH2 and treatment outcome. CDC2 is a mitotic cyclin-dependent Ser/Thr protein kinase and the master controller of mammalian cell–cycle regulation which is activated by CDK7 phosphorylation (31, 32). At present, expression of CDC2 has been linked to response to tamoxifen in cell line models (33), and we now show an 8-month delay in disease progression in patients with the lowest CDC2 mRNA levels compared with those with the highest expression levels. Thus, the status of the cell cycle regulation pathway, specified by CCNE1 and CDC2 levels but also confirmed by CCNB1, CCNE2, and E2F1, seems to play a role in how the metastasis will respond to first-line tamoxifen therapy.

Multivariate analysis of miR-26a, EZH2, CCNE1, and CDC2 to determine their associations with treatment outcome showed that the predictive values of EZH2 and CCNE1 levels were less significant than those of miR-26a and CDC2. Interestingly, not only miR-26a but also CDC2 have a physical interaction with EZH2 (Figure 3), although with opposite effects on EZH2 functioning. As mentioned, miR-26a binds to the 3'-UTR of EZH2 and inhibits transcription of EZH2. On the other hand, CDC2 (CDK1) and CDK2 have been shown to activate EZH2 by phosphorylation of its Thr350 residue (34-36). This Thr350 phosphorylation is necessary for EZH2 recruitment at target loci and for maintenance of H2K27me3 levels (34). Since EZH2 expression and activity are higher in proliferating rather than differentiating cells (22), both miR-26a and CDC2 may define endocrine-responsive or -resistant phenotypes of ER-positive breast cancer cells through their modulation of EZH2 levels and activity. In ER-negative breast cancer cells, EZH2 knockdown results in increased CDC2 and pCDC2 protein expressions (37), but recently it was suggested that EZH2 in ER-negative tumors functions as a transcriptional activator but acts as a repressor in ER-positive tumors (38).

Therapeutics that can modulate miR-26a, CDC2, or EZH2 activity might be an attractive strategy for patients resistant to tamoxifen to resensitize them for anti-estrogen treatment. Systemic administration of miR-26a with adeno-associated virus in mouse models results in decreased cancer cell proliferation and suppressed tumor progression (23). Preclinical evaluation of CDC2 and CDK2 inhibitors revealed G2/M arrest and cell death in both anti-estrogen-sensitive and resistant cells (33). Hydrolase inhibitors, such as DZNep, induce EZH2 depletion in breast cancer cell lines and result in cell cycle arrest and apoptosis (39, 40). At the end, all these treatments target EZH2 levels and activity. We hypothesize that patients resistant to tamoxifen with low miR-26a and high CDC2 and EZH2 levels in their primary tumor may benefit from these treatment strategies in order to overcome tamoxifen resistance.

In summary, we have shown that high miR-26a and low EZH2 mRNA levels associate with clinical benefit and prolonged TTP. The cell cycle regulation pathway and its genes CCNE1 and CDC2 correlate significantly with miR-26a and EZH2 levels and with outcome on tamoxifen. Multivariate analysis revealed miR-26a and CDC2 as sets of biomarkers to predict outcome on tamoxifen in metastatic breast cancer. Our findings might help one to improve the identification of individual patients resistant to tamoxifen, who may benefit from therapeutics that block EZH2 expression and activity.

FIGURE 3. The regulatory network of EZH2. A model for the modulation of the expression and activity of EZH2 based on our results and available data in the literature. Binding of miR-101 and miR-26a to the 3'-UTR blocks transcription of EZH2 (10, 11). Our data linked expression levels of miR-26a and EZH2 by the GTA of pathways to the cyclins and cell cycle regulation pathway with two significant genes [CCNE1 and CDC2 (CDK1)]. CDC2 (CDK1) and CDK2 activate EZH2 through the phosphorylation of its Thr350 residue (34-36). Our study shows that, in breast cancer, miR-26a and CDC2 might be involved in the regulation EZH2 expression and activity, respectively, and as a result associate with response to tamoxifen.



REFERENCES

1. Jansen MP, Foekens JA, van Staveren IL, Dirkzwager-Kiel MM, Ritstier K, Look MP, et al. Molecular classification of tamoxifen-resistant breast carcinomas by gene expression profiling. J Clin Oncol. 2005;23(4):732-40.
2. Kok M, Linn SC, Van Laar RK, Jansen MP, van den Berg TM, Delahaye LJ, et al. Comparison of gene expression profiles predicting progression in breast cancer patients treated with tamoxifen. Breast cancer research and treatment. 2009;113(2):275-83.
3. Schuettengruber B, Chourrout D, Vervoort M, Leblanc B, Cavalli G. Genome regulation by polycomb and trithorax proteins. Cell. 2007;128(4):735-45.
4. Reijm EA, Jansen MP, Ruigrok-Ritstier K, van Staveren IL, Look MP, van Gelder ME, et al. Decreased expression of EZH2 is associated with upregulation of ER and favorable outcome to tamoxifen in advanced breast cancer. Breast cancer research and treatment. 2011;125(2):387-94.
5. Wahid F, Shehzad A, Khan T, Kim YY. MicroRNAs: Synthesis, mechanism, function, and recent clinical trials. Biochim Biophys Acta. 2010.
6. Foekens JA, Sieuwerts AM, Smid M, Look MP, de Weerd V, Boersma AW, et al. Four miRNAs associated with aggressiveness of lymph node-negative, estrogen receptor-positive human breast cancer. Proceedings of the National Academy of Sciences of the United States of America. 2008;105(35):13021-6.
7. Verghese ET, Hanby AM, Speirs V, Hughes TA. Small is beautiful: microRNAs and breast cancer-where are we now? The Journal of pathology. 2008;215(3):214-21.
8. Miller TE, Ghoshal K, Ramaswamy B, Roy S, Datta J, Shapiro CL, et al. MicroRNA-221/222 confers tamoxifen resistance in breast cancer by targeting p27Kip1. The Journal of biological chemistry. 2008;283(44):29897-903.
9. Rodriguez-Gonzalez FG, Sieuwerts AM, Smid M, Look MP, Meijer-van Gelder ME, de Weerd V, et al. MicroRNA-30c expression level is an independent predictor of clinical benefit of endocrine therapy in advanced estrogen receptor positive breast cancer. Breast cancer research and treatment. 2011;127(1):43-51.
10. Varambally S, Cao Q, Mani RS, Shankar S, Wang X, Ateeq B, et al. Genomic loss of microRNA-101 leads to overexpression of histone methyltransferase EZH2 in cancer. Science (New York, NY. 2008;322(5908):1695-9.
11. Wong CF, Tellam RL. MicroRNA-26a targets the histone methyltransferase Enhancer of Zeste homolog 2 during myogenesis. The Journal of biological chemistry. 2008;283(15):9836-43.
12. Hayward JL, Carbone PP, Heuson JC, Kumaoka S, Segaloff A, Rubens RD. Assessment of response to therapy in advanced breast cancer: a project of the Programme on Clinical Oncology of the International Union Against Cancer, Geneva, Switzerland. Cancer. 1977;39(3):1289-94.
13. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM. REporting recommendations for tumor MARKer prognostic studies (REMARK). Breast cancer research and treatment. 2006;100(2):229-35.

14. Foekens JA, Portengen H, van Putten WL, Peters HA, Krijnen HL, Alexieva-Figusch J, et al. Prognostic value of estrogen and progesterone receptors measured by enzyme immunoassays in human breast tumor cytosols. Cancer research. 1989;49(21):5823-8.
15. Sieuwerts AM, Meijer-van Gelder ME, Timmermans M, Trapman AM, Garcia RR, Arnold M, et al. How ADAM-9 and ADAM-11 differentially from estrogen receptor predict response to tamoxifen treatment in patients with recurrent breast cancer: a retrospective study. Clin Cancer Res. 2005;11(20):7311-21.
16. Bachmann IM, Halvorsen OJ, Collett K, Stefansson IM, Straume O, Haukaas SA, et al. EZH2 expression is associated with high proliferation rate and aggressive tumor subgroups in cutaneous melanoma and cancers of the endometrium, prostate, and breast. J Clin Oncol. 2006;24(2):268-73.
17. Kleer CG, Cao Q, Varambally S, Shen R, Ota I, Tomlins SA, et al. EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells. Proceedings of the National Academy of Sciences of the United States of America. 2003;100(20):11606-11.
18. Cao P, Deng Z, Wan M, Huang W, Cramer SD, Xu J, et al. MicroRNA-101 negatively regulates Ezh2 and its expression is modulated by androgen receptor and HIF-1alpha/HIF-1beta. Mol Cancer. 2010;9:108.
19. Maillot G, Lacroix-Triki M, Pierredon S, Gratadou L, Schmidt S, Benes V, et al. Widespread estrogen-dependent repression of micrornas involved in breast tumor cell growth. Cancer research. 2009;69(21):8332-40.
20. Sachdeva M, Wu H, Ru P, Hwang L, Trieu V, Mo YY. MicroRNA-101-mediated Akt activation and estrogen-independent growth. Oncogene. 2011;30(7):822-31.
21. Czermin B, Melfi R, McCabe D, Seitz V, Imhof A, Pirrotta V. Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. Cell. 2002;111(2):185-96.
22. Margueron R, Li G, Sarma K, Blais A, Zavadil J, Woodcock CL, et al. Ezh1 and Ezh2 maintain repressive chromatin through different mechanisms. Molecular cell. 2008;32(4):503-18.
23. Kota J, Chivukula RR, O'Donnell KA, Wentzel EA, Montgomery CL, Hwang HW, et al. Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. Cell. 2009;137(6):1005-17.
24. Planas-Silva MD, Weinberg RA. Estrogen-dependent cyclin E-cdk2 activation through p21 redistribution. Mol Cell Biol. 1997;17(7):4059-69.
25. Osborne CK, Boldt DH, Clark GM, Trent JM. Effects of tamoxifen on human breast cancer cell cycle kinetics: accumulation of cells in early G1 phase. Cancer research. 1983;43(8):3583-5.
26. Sieuwerts AM, Look MP, Meijer-van Gelder ME, Timmermans M, Trapman AM, Garcia RR, et al. Which cyclin E prevails as prognostic marker for breast cancer? Results from a retrospective study involving 635 lymph node-negative breast cancer patients. Clin Cancer Res. 2006;12(11 Pt 1):3319-28.

27. Waltersson MA, Askmalm MS, Nordenskjold B, Fornander T, Skoog L, Stal O. Altered expression of cyclin E and the retinoblastoma protein influences the effect of adjuvant therapy in breast cancer. Int J Oncol. 2009;34(2):441-8.
28. Akli S, Zheng PJ, Multani AS, Wingate HF, Pathak S, Zhang N, et al. Tumor-specific low molecular weight forms of cyclin E induce genomic instability and resistance to p21, p27, and antiestrogens in breast cancer. Cancer research. 2004;64(9):3198-208.
29. Akli S, Bui T, Wingate H, Biernacka A, Moulder S, Tucker SL, et al. Low-molecular-weight cyclin E can bypass letrozole-induced G1 arrest in human breast cancer cells and tumors. Clin Cancer Res. 2010;16(4):1179-90.
30. Bagheri-Yarmand R, Nanos-Webb A, Biernacka A, Bui T, Keyomarsi K. Cyclin E deregulation impairs mitotic progression through premature activation of Cdc25C. Cancer research. 2010;70(12):5085-95.
31. Satyanarayana A, Kaldis P. Mammalian cell-cycle regulation: several Cdks, numerous cyclins and diverse compensatory mechanisms. Oncogene. 2009;28(33):2925-39.
32. Malumbres M, Barbacid M. Cell cycle, CDKs and cancer: a changing paradigm. Nat Rev Cancer. 2009;9(3):153-66.
33. Johnson N, Bentley J, Wang LZ, Newell DR, Robson CN, Shapiro GI, et al. Pre-clinical evaluation of cyclin-dependent kinase 2 and 1 inhibition in anti-estrogen-sensitive and resistant breast cancer cells. British journal of cancer. 2010;102(2):342-50.
34. Chen S, Bohrer LR, Rai AN, Pan Y, Gan L, Zhou X, et al. Cyclin-dependent kinases regulate epigenetic gene silencing through phosphorylation of EZH2. Nat Cell Biol. 2010;12(11):1108-14.
35. Wei Y, Chen YH, Li LY, Lang J, Yeh SP, Shi B, et al. CDK1-dependent phosphorylation of EZH2 suppresses methylation of H3K27 and promotes osteogenic differentiation of human mesenchymal stem cells. Nat Cell Biol. 2011;13(1):87-94.
36. Kaneko S, Li G, Son J, Xu CF, Margueron R, Neubert TA, et al. Phosphorylation of the PRC2 component Ezh2 is cell cycle-regulated and up-regulates its binding to ncRNA. Genes & development. 2010;24(23):2615-20.
37. Gonzalez ME, Li X, Toy K, DuPrie M, Ventura AC, Banerjee M, et al. Downregulation of EZH2 decreases growth of estrogen receptor-negative invasive breast carcinoma and requires BRCA1. Oncogene. 2009;28(6):843-53.
38. Lee ST, Li Z, Wu Z, Aau M, Guan P, Karuturi RK, et al. Context-specific regulation of NF-kappaB target gene expression by EZH2 in breast cancers. Molecular cell. 2011;43(5):798-810.
39. Hayden A, Johnson PW, Packham G, Crabb SJ. S-adenosylhomocysteine hydrolase inhibition by 3-deazaneplanocin A analogues induces anti-cancer effects in breast cancer cell lines and synergy with both histone deacetylase and HER2 inhibition. Breast cancer research and treatment. 2011;127(1):109-19.
40. Tan J, Yang X, Zhuang L, Jiang X, Chen W, Lee PL, et al. Pharmacologic disruption of Polycomb-repressive complex 2-mediated gene repression selectively induces apoptosis in cancer cells. Genes Dev. 2007;21(9):1050-63.
41. Smid M, Wang Y, Zhang Y, Sieuwerts AM, Yu J, Klijn JG, et al. Subtypes of breast cancer show preferential site of relapse. Cancer research. 2008;68(9):3108-14.

SUPPLEMENTARY MATERIAL

https://drive.google.com/open?id=0B_eW3FoE0HzYdEJNZHlUcWx6ODg

CHAPTER 4

High protein expression of EZH2 is related to unfavorable outcome to tamoxifen in metastatic breast cancer

E.A. Reijm, A.M. Timmermans, M.P. Look, M.E. Meijer-van Gelder, C.H.M. van Deurzen, J.W.M. Martens, S. Sleijfer, J.A. Foekens, P.M.J.J. Berns, and M.P.H.M. Jansen

Ann Oncol 2014 Nov; 25(11): 2185-90

ABSTRACT

BACKGROUND

Metastatic breast cancer (MBC) is a highly heterogeneous disease with great differences in outcome to both chemo- and endocrine therapy. Better insight into the mechanisms underlying resistance is essential to better predict outcome to therapy and to obtain a more tailored treatment approach. We have previously described that increased mRNA expression levels of Enhancer of Zeste homolog (EZH2) are associated with worse outcome to tamoxifen therapy in MBC. Here, we explored whether this is also the case for EZH2 protein expression.

PATIENTS AND METHODS

A tissue microarray (TMA) was created using formalin-fixed, paraffin-embedded estrogen receptor (ER)-positive primary breast tumor tissues of 250 MBC patients treated with first-line tamoxifen. Quantity and intensity of EZH2 expression were determined by immunohistochemistry (IHC) and both were used to generate and group scores according to a previously described method for scoring EZH2.

RESULTS

In total, 116 tumors (46%) were considered to be EZH2 positive. The presence of EZH2 protein expression was significantly associated with progression-free survival (PFS) in both univariate [hazard ratio (HR) 1.51, 95% confidence interval (CI) 1.17–1.97, P = 0.002] and multivariate analysis including traditional factors associated with tamoxifen outcome (HR 1.41, 95% CI 1.06–1.88, P = 0.017). Considering quantity irrespective of intensity, tumors with >50% EZH2-positive cells had the worst PFS (HR 2.15, 95% CI 1.42–3.27, P < 0.001), whereas intensity alone did not show a significant association with PFS. Application of other methods of scoring EZH2 positivity resulted in a similar significant association between the amount of EZH2 positive cells and PFS.

CONCLUSION

In addition to EZH2 mRNA levels, these results suggest that protein expression of EZH2 can be used as a marker to predict outcome to tamoxifen therapy. This provides new rationale to explore EZH2 inhibition in the clinical setting and increases the possibilities for a more personalized treatment approach in MBC patients.

INTRODUCTION

For already more than 30 years, both primary and metastatic breast cancer (MBC) patients with estrogen receptor (ER)-expressing tumors greatly benefit from endocrine therapies such as tamoxifen. However, outcome to tamoxifen differs considerably between MBC patients with ~30% not benefitting at all while virtually all patients who initially benefit eventually develop progressive disease due to acquired resistance. Consequently, there is a high need to get more insight into the factors causing resistance to tamoxifen in order to develop methods to overcome resistance and to identify biomarkers to obtain a more personalized treatment approach. Numerous factors have already been revealed to account for resistance to endocrine therapy including loss of ER expression (1-3), overexpression of the HER2 receptor (4), and hyperactivation of the phosphatidylinositol 3-kinase (PI3K) pathway (5), and have subsequently led to the development of new targeted therapies (6, 7). Additionally, we have previously identified overexpression of EZH2 as a potential factor involved in therapy resistance (8, 9). EZH2, being a histone methyltransferase that mediates di- and trimethylation of lysine residue 27 on histone 3 (H3K27), comprises the catalytic subunit of the Polycomb Repressor Complex 2 (PRC2) and belongs to the Polycomb group (PcG) proteins. These proteins mainly act as transcriptional repressors of multiple genes involved in embryonic development, cell differentiation, and carcinogenesis (10-12). EZH2 has been found to play a pivotal role in the tumorigenic process since its expression has been shown to be upregulated in several malignancies including prostate cancer (13), lymphomas (14, 15), urogenital tract tumors (16-18), and breast cancer (19). Concerning the latter, increased expression of EZH2 protein has been associated with a high histological grade and worse survival suggesting its promising role as a prognostic biomarker for aggressive breast cancer (18-21). Additionally, EZH2 has been proposed as a therapeutic target due to its association with tumor aggressiveness in several tumor types (18-23). Recently, small-molecule inhibitors of EZH2 histone methyltransferase activity have showed to decrease global H3K27me3 levels resulting in reactivation of silenced target genes and inhibition of proliferation, particularly in lymphomas with EZH2-activating mutations (24). In breast cancer, we have previously shown that low expression of EZH2 mRNA was associated with a favorable response to tamoxifen treatment in MBC patients as well as, using *in vitro* silencing techniques, in a preclinical setting, which suggests EZH2 as a new potential therapeutic target (9). However, this study was carried out on mRNA expression levels of EZH2 while the predictive value of EZH2 on protein expression level has not yet been studied. In the current study, we explored the association between protein expression of EZH2 and PFS in tamoxifen-treated MBC patients.

PATIENTS AND METHODS

ETHICS STATEMENT

This retrospective study, in which coded tumor tissues were used, has been approved by the medical ethics committee of the Erasmus MC Rotterdam, The Netherlands (MEC 02.953). Informed consent was not required. The study was carried out in accordance with the Code of Conduct of the Federation of Medical Scientific Societies in The Netherlands (http://www.fmwv.nl) and has been reported, wherever possible, following the REMARK guidelines (25).

PATIENTS AND TUMOR TISSUES

Patients with primary operable ER-positive breast cancer between 1985 and 1998 and from whom a formalin-fixed, paraffin-embedded primary breast tumor tissue specimen was available were included in the study provided that they subsequently developed MBC treated with first-line tamoxifen and detailed clinical follow-up data were available. In order to be included for final analysis, tumor specimens needed to have sufficient tumor material to obtain three independent cores. Central pathology review of the primary tumor was carried out by a breast pathologist (CvD), which included histologic subtype according to the World Health Organization (WHO) and histologic grade defined according to the modified Bloom–Richardson score based on the percentage of tubule formation, nuclear pleomorphism, and mitotic activity (26). After applying the inclusion criteria of having three independent tumor cores per patient, tumor core biopsies on a breast tissue microarray (TMA) (for details see supplementary materials and methods) for 250 of the 316 available patients were selected for further analyses. Of these 250 patients, 106 (42%) underwent breast conserving surgery and 144 modified mastectomy (58%). Median follow-up time was 98 months (range 9–222 months). One hundred fifty-three patients (61%) did not receive adjuvant systemic therapy, while 77 (31%) were treated with adjuvant chemotherapy and 20 (8%) had metastatic disease upon diagnosis. None of the patients received adjuvant hormonal therapy. Response to first-line tamoxifen treatment, as assessed by RECIST criteria (version 1.1; http://www.eortc.be/recist/), was observed in 156 patients (62%) of whom 7 had complete response (CR; 3%), 42 a partial response (PR; 17%), and 107 (43%) stable disease for more than 6 months. Ninety-four patients (38%) did not have clinical benefit.

PFS was used as end point with PFS defined as the time elapsed between start of tamoxifen therapy and the first detection of disease progression or death, whatever came first during treatment. Detailed clinicopathological information for these 250 patients is provided in Supplementary Table S1.

IMMUNOHISTOCHEMICAL EVALUATION

EZH2 staining was interpreted and recorded independently by two experienced observers (EAR and AMT) in a blinded manner. Expression was assessed using a combination of both intensity (0 no staining, 1 weak, 2 moderate, and 3 strong) and proportion of cells stained for EZH2 (1 ≤10%, 2 11%–50%, and 3 ≥50%) according to the method described previously by Bachmann et al. (18). Scores were multiplied to a maximum of 9 and a cutoff of >3 was considered as EZH2 positive.

DATA ANALYSIS AND STATISTICS

Statistical analyses were done with the STATA statistical package, release 12.0 (STATA Corp., College Station, TX). The relationship of EZH2 expression with patient and tumor characteristics was investigated using Pearson's χ^2 test or Fisher's exact when expected numbers were too small. The Cox proportional hazards model was used to compute the hazard ratio (HR) with the 95% CI in the analysis of PFS. In multivariate analysis, logistic and Cox regression analysis were used to determine whether the intensity and quantity of EZH2 expression had predictive value and were independent when added to the base model of traditional factors. Survival curves were generated using the Kaplan–Meier method and a log-rank test was used to test for differences between survival curves. When more than 2 curves were compared a log-rank test for trend was used for ordered categories. All P values were two-sided and $P < 0.05$ was considered statistically significant.

RESULTS

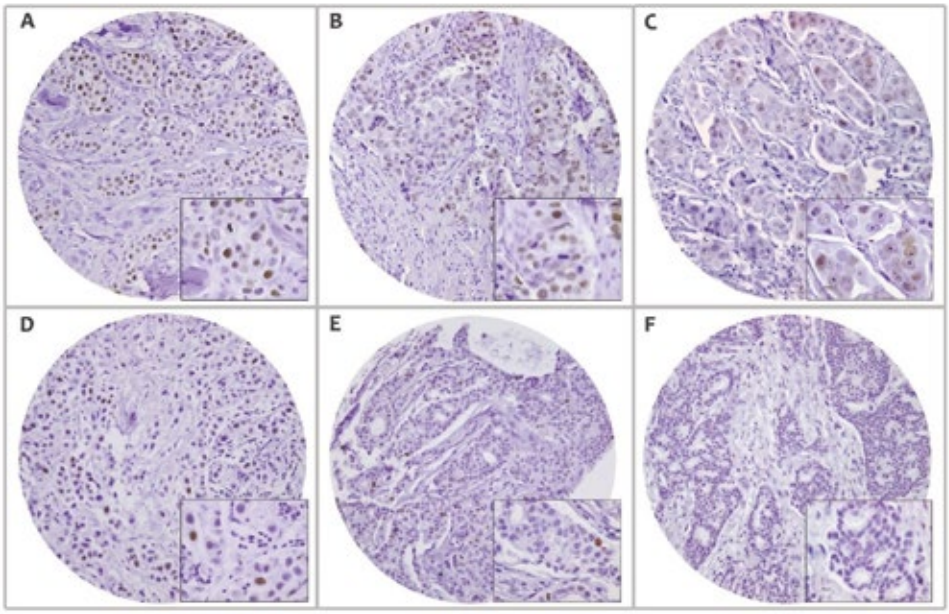
QUANTITY AND INTENSITY OF EZH2 PROTEIN EXPRESSION

EZH2 expression was primarily identified in the nucleus, as reported previously (14, 19, 21, 27). Fifty-one of the 250 tumors (20%) did not show any EZH2 expression (Supplementary Table S2). Together with the 66 tumors (26%) that showed weak staining irrespective of the percentage of stained cells and the 17 tumors (7%) that showed moderate staining in <10% of the cells these were classified as EZH2 negative. The 116 remaining tumors (46%) were ranked as EZH2 positive, 93 showed moderate staining in >10% of the cells while 23 were strongly stained (Supplementary Table S2, Figure 1).

ASSOCIATION BETWEEN EZH2 PROTEIN EXPRESSION WITH CLINICOPATHOLOGICAL CHARACTERISTICS

There was no significant association between EZH2 protein expression and menopausal status, tumor histology, progesterone receptor (PgR) status, dominant site of relapse (DSR), and disease-free interval (DFI) (Supplementary Table S1). A significant positive association was observed with age, the amount of lymph nodes involved at initial diagnosis, histologic grade, and HER2/neu status.

FIGURE 1. Representative tissues with IHC staining of EZH2 protein. Strong (A), moderate (B), and weak (C) nuclear staining in >50% of the tumor cells. (D) Strong nuclear staining in 21%-30% of the cells. (E) Moderate nuclear staining in 6-10% of the cells. (F) Negative staining of EZH2 protein.A, B, and D obtained a score of >3 and were classified as EZH2 positive; C, E and F were considered to be EZH2 negative.



ASSOCIATION OF EZH2 PROTEIN EXPRESSION WITH PFS

In univariate analysis, the presence of EZH2 protein expression was significantly associated with PFS (HR 1.51, 95% CI 1.17–1.97, $P = 0.002$) (Table 1 and Figure 2A). When considering intensity and quantity separately, intensity showed no relation with PFS (Figure 2B). Quantity was associated with PFS with tumors with >50% EZH2-positive cells ($N = 31$) having the poorest PFS (HR 2.15, 95% CI 1.42–3.27, $P < 0.001$) (Table 1 and Figure 2C). In multivariate analysis, when corrected for traditional predictive factors including age, menopausal status, adjuvant therapy, DSR, DFI, PgR, and HER2/neu status, tumors with EZH2 protein expression were significantly associated with poor PFS (HR 1.41, 95% CI 1.06–1.88, $P = 0.017$) compared with tumors that scored negative for EZH2 protein expression. The overall survival data showed a HR of 1.31 with 95% CI 0.99–1.73, $P = 0.055$.

TABLE 1. Cox uni- and multivariate analysis for PFS of EZH2 protein expression levels for the product of intensity and quantity and separate analysis of intensity and quantity in 250 ER-positive tumors from patients with MBC treated with first-line tamoxifen therapy.

Factor of base model		**Univariate analysis**			**Multivariate analysis**		
	N	**HR**	**95% CI**	***P***	**HR**	**95% CI**	***P***
Age (years)							
≤40	14	1.00			1.00		
41-55	76	1.12	0.62-2.02	0.710	1.13	0.61-2.11	0.699
56-70	92	0.69	0.38-1.23	0.208	0.57	0.27-1.22	0.150
>70	68	0.54	0.29-0.99	0.046	0.46	0.20-1.02	0.056
Menopausal status							
Premenopausal	64	1.00			1.00		
Postmenopausal	186	0.64	0.48-0.85	0.003	1.12	0.70-1.78	0.644
Adjuvant therapy							
None	173*	1.00			1.00		
Chemotherapy	77	1.38	1.04-1.82	0.023	0.97	0.69-1.37	0.853
Disease-free interval (years)							
≤1	38	1.00			1.00		
1-3	118	0.86	0.59-1.25	0.430	0.71	0.48-1.07	0.105
>3	94	0.71	0.48-1.06	0.092	0.60	0.39-0.93	0.023
Dominant site of relapse							
Soft tissue	25	1.00			1.00		
Bone	127	1.51	0.93-2.43	0.093	1.41	0.87-2.31	0.166
Viscera	98	1.97	1.20-3.24	0.007	2.12	1.28-3.52	0.004
PgR†							
Negative	64	1.00			1.00		
Positive	188	0.88	0.65-1.17	0.376	0.80	0.59-1.08	0.146
HER2/neu†							
Negative	204	1.00			1.00		
Positive	46	1.09	0.78-1.53	0.620	0.94	0.66-1.35	0.739
Factors analyzed					**Additions to the base model**		
EZH2 protein	250	1.51	1.17-1.97	0.002	1.41	1.06-1.88	0.017
Intensity							
Negative	51	1.00					
Weak	66	0.90	0.61-1.32	0.581			
Moderate	110	1.28	0.90-1.81	0.171			
Strong	23	1.34	0.81-2.23	0.257			

TABLE 1. (continued)

Factor of base model		Univariate analysis			Multivariate analysis		
	N	HR	95% CI	*P*	HR	95% CI	*P*
Quantity (%)							
<10%	110	1.00			1.00		
11-50	109	1.46	1.10-1.92	0.008	1.45	1.07-1.96	0.017
>50	31	2.15	1.42-3.27	<0.001	2.03	1.30-3.16	0.002

* Combination of no adjuvant therapy (*N* = 153) and metastatic disease at diagnosis (*N* =20)
† As retrieved from TMA

FIGURE 2.

A. Kaplan-Meier curve of PFS as a function of EZH2. Patients were divided into two groups according to their expression levels of EZH2 in which EZH2 positivity was defined as >3 based on the scores of both intensity and quantity. Patients at risk at different time points are indicated.

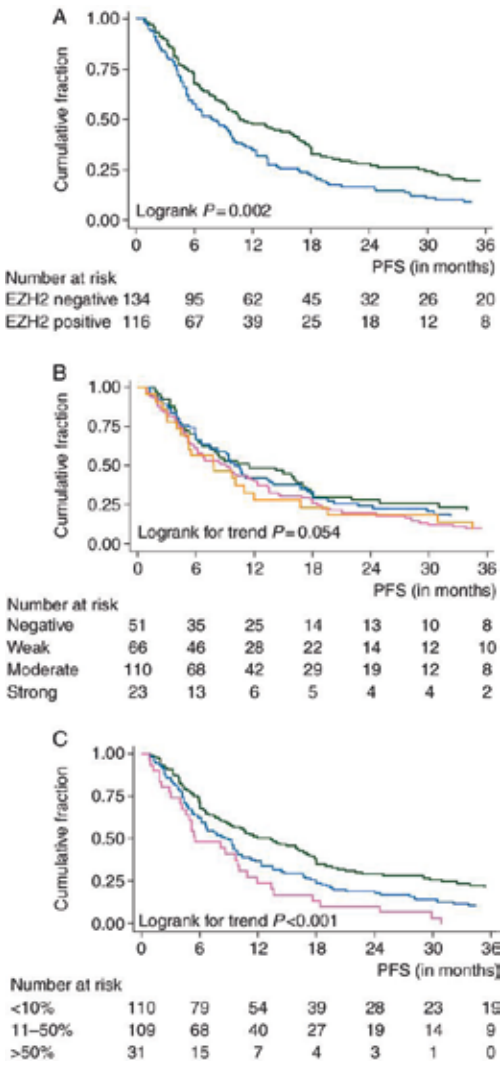
Green: EZH2 negative; Blue: EZH2 positive.

B. Kaplan-Meier curve of PFS as a function of intensity of EZH2. Patients were divided into four groups according to the intensity of EZH2 staining in their primary tumor tissue specimen. Patients at risk at different time points are indicated.

Green: no staining; Blue: weak; Red: moderate; Orange: strong.

C. Kaplan-Meier curve of PFS as a function of quantity of EZH2. Patients were divided into three groups according to the amount of EZH2-positive cells in their primary tumor tissue specimen. Patients at risk at different time points are indicated.

Green: <10%; Blue: 11-50%; Red: >50%.



DISCUSSION

In the present study, we demonstrate the association between high levels of protein expression of EZH2 and poor outcome to endocrine therapy in ER-positive MBC patients treated with first-line tamoxifen. To our best knowledge, this is the first study that shows a relationship between EZH2 protein expression and outcome to tamoxifen therapy in MBC. These results are concordant with our previous study showing a similar association based on EZH2 mRNA levels (9). There, we also found an association between high EZH2 mRNA expression and lower clinical benefit. In this study, we could establish similar results on protein level with an association between EZH2 protein expression and a worse response rate, defined as CR and PR after initiation of therapy (odds ratio 0.17, 95% CI 0.10–0.29, $P < 0.001$; data not shown). Of the 250 patients in this study, we had EZH2 mRNA data available of 66 patients (26%). In this small subgroup, we observed a significant positive correlation between presence of EZH2 protein and EZH2 mRNA ($P = 0.04$) (Supplementary Figure S1).

EZH2 has previously been suggested to bear prognostic value in several types of cancer. By using the same technique of TMAs, Alford et al. reported higher EZH2 expression in invasive breast carcinomas that relapsed after primary diagnosis compared with those that did not, regardless of received systemic adjuvant therapy (27). As expected in our study of ER-positive tumors, most of the tumors were classified as moderately differentiated. The poorly differentiated tumors were more often EZH2 positive compared with the well and moderately differentiated. In accordance with our findings, an association between high EZH2 expression and high histologic grade has been demonstrated multiple times despite the use of different methods to classify the differentiation of tumors (18, 20, 21).

Importantly, the assessment of scoring protein expression is semiquantitative and several methods exist. One of the most widely used methods to evaluate nuclear immunostaining is the Allred score in which quantity and intensity are also involved but quantity is composed of six categories (28). This complex method is convenient to use for commonly expressed factors as ER, EGFR, and PgR (29-31), but it is difficult to divide less frequently expressed factors such as EZH2 into the six different quantity categories. Since there is no golden standard for scoring EZH2, we used the method of Bachmann et al. (18) in which the product of intensity and quantity is used and a cutoff value of >3 to quantify tumors as EZH2 positive. However, other studies on EZH2 protein expression in breast cancer used different criteria. Kleer et al. (19) and Alford et al. (27) only used quantity when scoring 194 and 480 invasive carcinomas, respectively, and classified tumors with >25% of nuclei staining with any intensity as EZH2 positive. Raaphorst et al. (21) also judged only quantity without a cutoff for positivity in 25 breast tumors ranging from precursor lesions to invasive tumors. In our study, adapted application of the scores as used by Kleer et al. (19), Alford et al. (27), and Raaphorst et al. (21) also resulted in an association between EZH2 protein expression and PFS despite of the difference in the number of tumors that were classified as EZH2 positive when applying the different methods (116 tumors using the method of Bachmann et al. versus 105 tumors using

Kleer et al. and Alford et al., respectively) (Supplementary Figure S2A and B and Tables S3A and B and S4). Due to lack of standardized methods and to have a validation, we have also applied the method of Chan et al. to validate the different methods of EZH2 scoring (32). This method has been used to classify the expression of the seven in absentia homolog 2 (SIAH2) protein in breast tumors and uses a sum of intensity and quantity with a cutoff value of >2 to quantify tumors as SIAH2 positive. Application of this method to EZH2 scoring resulted in a similar association between EZH2 protein expression and PFS (Supplementary Figure S2C and Tables S3C and S4). Thus, the relation seems to be independent of the method of scoring, as long as the amount proportion of stained cells is included since intensity by itself did not associate with PFS, whereas quantity did according to different methods.

Considering downstream effects of EZH2, we have previously shown that silencing of EZH2 in the ER-positive cell line MCF7 leads to a higher expression of ER and increased sensitivity to antiestrogen treatment (9). The present study confirms this association between EZH2 and sensitivity to tamoxifen by demonstrating the association between high EZH2 protein expression and poor outcome to first-line tamoxifen treatment in MBC patients. Therefore, adding EZH2 inhibition to antiestrogen therapies is an approach worthwhile to explore in MBC and EZH2 expression might be the biomarker to select patients who benefit most.

Recently, several EZH2 inhibitors have been developed and preclinically tested in multiple types of cell lines and xenografts (33, 34). Both EPZ-6438 (E7438) and GSK126 as selective small-molecule inhibitors of histone methyltransferase activity have shown promising results in both small-cell lung cancer (SCLC) cell lines and malignant rhabdoid tumors xenografts. The most convincing preclinical results have been obtained in lymphomas, which often bear EZH2-activating mutations (24). This has led to the recent start of a phase I/II trial in patients with EZH2-mutated B-cell lymphomas (www.clinicaltrials.gov, NCT01897571). EZH2 mutations have not yet been described in other types of tumors.

In conclusion, we demonstrate that high EZH2 protein levels are associated with unfavorable outcome to tamoxifen treatment in MBC patients. We explored multiple methods for scoring EZH2 protein levels leading to a preference for the use of quantity or the sum or product of intensity and quantity over intensity alone due to their significant association with PFS. The association of EZH2 expression, both at the mRNA and the protein level, with outcome to tamoxifen in MBC and the previous finding that downregulation of EZH2 increases ER expression and subsequently, sensitivity to anti-estrogen treatment in preclinical models renders EZH2 an attractive target to explore in combination with anti-estrogen treatment in ER-positive breast cancer. Furthermore, its assessment could be used to categorize patients according to their likelihood to benefit from tamoxifen and if confirmed, could allow a better selection of patients for tamoxifen and a more personalized treatment approach.

REFERENCES

1. Thompson AM, Jordan LB, Quinlan P, Anderson E, Skene A, Dewar JA, et al. Prospective comparison of switches in biomarker status between primary and recurrent breast cancer: the Breast Recurrence In Tissues Study (BRITS). Breast Cancer Res. 2010;12(6):R92.
2. Gong Y, Han EY, Guo M, Pusztai L, Sneige N. Stability of estrogen receptor status in breast carcinoma: a comparison between primary and metastatic tumors with regard to disease course and intervening systemic therapy. Cancer. 2011;117(4):705-13.
3. Amir E, Miller N, Geddie W, Freedman O, Kassam F, Simmons C, et al. Prospective study evaluating the impact of tissue confirmation of metastatic disease in patients with breast cancer. J Clin Oncol. 2012;30(6):587-92.
4. Pancholi S, Lykkesfeldt AE, Hilmi C, Banerjee S, Leary A, Drury S, et al. ERBB2 influences the subcellular localization of the estrogen receptor in tamoxifen-resistant MCF-7 cells leading to the activation of AKT and RPS6KA2. Endocr Relat Cancer. 2008;15(4):985-1002.
5. Miller TW, Balko JM, Arteaga CL. Phosphatidylinositol 3-kinase and antiestrogen resistance in breast cancer. J Clin Oncol. 2011;29(33):4452-61.
6. Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med. 2001;344(11):783-92.
7. Hong DS, Bowles DW, Falchook GS, Messersmith WA, George GC, O'Bryant CL, et al. A multicenter phase I trial of PX-866, an oral irreversible phosphatidylinositol 3-kinase inhibitor, in patients with advanced solid tumors. Clin Cancer Res. 2012;18(15):4173-82.
8. Jansen MP, Foekens JA, van Staveren IL, Dirkzwager-Kiel MM, Ritstier K, Look MP, et al. Molecular classification of tamoxifen-resistant breast carcinomas by gene expression profiling. J Clin Oncol. 2005;23(4):732-40.
9. Reijm EA, Jansen MP, Ruigrok-Ritstier K, van Staveren IL, Look MP, van Gelder ME, et al. Decreased expression of EZH2 is associated with upregulation of ER and favorable outcome to tamoxifen in advanced breast cancer. Breast Cancer Res Treat. 2011;125(2):387-94.
10. Bracken AP, Dietrich N, Pasini D, Hansen KH, Helin K. Genome-wide mapping of Polycomb target genes unravels their roles in cell fate transitions. Genes Dev. 2006;20(9):1123-36.
11. Schuettengruber B, Chourrout D, Vervoort M, Leblanc B, Cavalli G. Genome regulation by polycomb and trithorax proteins. Cell. 2007;128(4):735-45.
12. Cao R, Wang L, Wang H, Xia L, Erdjument-Bromage H, Tempst P, et al. Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science. 2002;298(5595):1039-43.
13. Varambally S, Dhanasekaran SM, Zhou M, Barrette TR, Kumar-Sinha C, Sanda MG, et al. The polycomb group protein EZH2 is involved in progression of prostate cancer. Nature. 2002;419(6907):624-9.
14. Raaphorst FM, van Kemenade FJ, Blokzijl T, Fieret E, Hamer KM, Satijn DP, et al. Coexpression of BMI-1 and EZH2 polycomb group genes in Reed-Sternberg cells of Hodgkin's disease. Am J Pathol. 2000;157(3):709-15.

15. Visser HP, Gunster MJ, Kluin-Nelemans HC, Manders EM, Raaphorst FM, Meijer CJ, et al. The Polycomb group protein EZH2 is upregulated in proliferating, cultured human mantle cell lymphoma. Br J Haematol. 2001;112(4):950-8.
16. Raman JD, Mongan NP, Tickoo SK, Boorjian SA, Scherr DS, Gudas LJ. Increased expression of the polycomb group gene, EZH2, in transitional cell carcinoma of the bladder. Clin Cancer Res. 2005;11(24 Pt 1):8570-6.
17. Weikert S, Christoph F, Kollermann J, Muller M, Schrader M, Miller K, et al. Expression levels of the EZH2 polycomb transcriptional repressor correlate with aggressiveness and invasive potential of bladder carcinomas. Int J Mol Med. 2005;16(2):349-53.
18. Bachmann IM, Halvorsen OJ, Collett K, Stefansson IM, Straume O, Haukaas SA, et al. EZH2 expression is associated with high proliferation rate and aggressive tumor subgroups in cutaneous melanoma and cancers of the endometrium, prostate, and breast. J Clin Oncol. 2006;24(2):268-73.
19. Kleer CG, Cao Q, Varambally S, Shen R, Ota I, Tomlins SA, et al. EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells. Proc Natl Acad Sci U S A. 2003;100(20):11606-11.
20. Collett K, Eide GE, Arnes J, Stefansson IM, Eide J, Braaten A, et al. Expression of enhancer of zeste homologue 2 is significantly associated with increased tumor cell proliferation and is a marker of aggressive breast cancer. Clin Cancer Res. 2006;12(4):1168-74.
21. Raaphorst FM, Meijer CJ, Fieret E, Blokzijl T, Mommers E, Buerger H, et al. Poorly differentiated breast carcinoma is associated with increased expression of the human polycomb group EZH2 gene. Neoplasia. 2003;5(6):481-8.
22. Eskander RN, Ji T, Huynh B, Wardeh R, Randall LM, Hoang B. Inhibition of Enhancer of Zeste Homolog 2 (EZH2) Expression Is Associated With Decreased Tumor Cell Proliferation, Migration, and Invasion in Endometrial Cancer Cell Lines. Int J Gynecol Cancer. 2013;23(6):997-1005.
23. Hubaux R, Thu KL, Coe BP, Macaulay C, Lam S, Lam WL. EZH2 Promotes E2F-Driven SCLC Tumorigenesis through Modulation of Apoptosis and Cell-Cycle Regulation. J Thorac Oncol. 2013;8(8):1102-6.
24. McCabe MT, Ott HM, Ganji G, Korenchuk S, Thompson C, Van Aller GS, et al. EZH2 inhibition as a therapeutic strategy for lymphoma with EZH2-activating mutations. Nature. 2012;492(7427):108-12.
25. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM, et al. REporting recommendations for tumor MARKer prognostic studies (REMARK). Breast Cancer Res Treat. 2006;100(2):229-35.
26. Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology. 1991;19(5):403-10.
27. Alford SH, Toy K, Merajver SD, Kleer CG. Increased risk for distant metastasis in patients with familial early-stage breast cancer and high EZH2 expression. Breast Cancer Res Treat. 2012;132(2):429-37.
28. Allred DC, Clark GM, Elledge R, Fuqua SA, Brown RW, Chamness GC, et al. Association of p53 protein expression with tumor cell proliferation rate and clinical outcome in node-

negative breast cancer. J Natl Cancer Inst. 1993;85(3):200-6.
29. Harvey JM, Clark GM, Osborne CK, Allred DC. Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. J Clin Oncol. 1999;17(5):1474-81.
30. Rokita M, Stec R, Bodnar L, Charkiewicz R, Korniluk J, Smoter M, et al. Overexpression of epidermal growth factor receptor as a prognostic factor in colorectal cancer on the basis of the Allred scoring system. Onco Targets Ther. 2013;6:967-76.
31. Henriksen KL, Rasmussen BB, Lykkesfeldt AE, Moller S, Ejlertsen B, Mouridsen HT. Semi-quantitative scoring of potentially predictive markers for endocrine treatment of breast cancer: a comparison between whole sections and tissue microarrays. J Clin Pathol. 2007;60(4):397-404.
32. Chan P, Moller A, Liu MC, Sceneay JE, Wong CS, Waddell N, et al. The expression of the ubiquitin ligase SIAH2 (seven in absentia homolog 2) is mediated through gene copy number in breast cancer and is associated with a basal-like phenotype and p53 expression. Breast Cancer Res. 2011;13(1):R19.
33. Sato T, Kaneda A, Tsuji S, Isagawa T, Yamamoto S, Fujita T, et al. PRC2 overexpression and PRC2-target gene repression relating to poorer prognosis in small cell lung cancer. Sci Rep. 2013;3:1911.
34. Knutson SK, Warholic NM, Wigle TJ, Klaus CR, Allain CJ, Raimondi A, et al. Durable tumor regression in genetically altered malignant rhabdoid tumors by inhibition of methyltransferase EZH2. Proc Natl Acad Sci U S A. 2013;110(19):7922-7.

SUPPLEMENTARY MATERIAL

https://drive.google.com/open?id=0B_eW3FoE0HzYdEJNZHlUcWx6ODg

CHAPTER 5

Hallmarks of Aromatase Inhibitor Drug Resistance Revealed by Epigenetic Profiling in Breast Cancer

M.P.H.M. Jansen, T. Knijnenburg, E.A. Reijm, I.M. Simon, R. Kerkhoven, M. Droog, A. Velds, S. van Laere, L.Y. Dirix, X. Alexi, J.A. Foekens, L. Wessels, S.C. Linn, E.M.J.J. Berns, and W. Zwart

Cancer Res 2013; 73(22): 6632-6641

ABSTRACT

Aromatase inhibitors are the major first-line treatment of estrogen receptor–positive breast cancer, but resistance to treatment is common. To date, no biomarkers have been validated clinically to guide subsequent therapy in these patients. In this study, we mapped the genome-wide chromatin-binding profiles of estrogen receptor α (ERα), along with the epigenetic modifications H3K4me3 and H3K27me3, that are responsible for determining gene transcription (*N* = 12). Differential binding patterns of ERα, H3K4me3, and H3K27me3 were enriched between patients with good or poor outcomes after aromatase inhibition. ERα and H3K27me3 patterns were validated in an additional independent set of breast cancer cases (*N* = 10). We coupled these patterns to array-based proximal gene expression and progression-free survival data derived from a further independent cohort of 72 aromatase inhibitor–treated patients. Through this approach, we determined that the ERα and H3K27me3 profiles predicted the treatment outcomes for first-line aromatase inhibitors. In contrast, the H3K4me3 pattern identified was not similarly informative. The classification potential of these genes was only partially preserved in a cohort of 101 patients who received first-line tamoxifen treatment, suggesting some treatment selectivity in patient classification.

INTRODUCTION

Breast cancer is the most frequently diagnosed malignancy among women worldwide, with annually around 1.4 million new cases and half a million patients who die from the disease each year (1). Seventy-five percent of all breast tumors are of the luminal subtype and tumor cell proliferation is thought to depend on activity of the estrogen receptor α (ERα). Inhibition of ERα by endocrine therapy is therefore a major treatment modality for these tumors, either by tamoxifen or the state-of-the-art aromatase inhibitors. Although many studies have focused on defining predictive markers for tamoxifen resistance, relatively little is known about the molecular determinants of aromatase inhibitor response. Such knowledge is essential as intrinsic and acquired resistance to treatment is common (2, 3). Whole-genome sequencing analyses on breast tumor samples revealed a set of 18 genes to be mutated in patients with breast cancer that correlated with differential survival upon aromatase inhibitor treatment (4), including PI3K, TP53, MAP3K1, and GATA3. These data were supported by other reports, indicating differential gene expression pathways to be enriched in poor versus good outcome patients upon aromatase inhibitor treatment (5, 6).

ERα–binding profile assessment in breast cancer cell lines has greatly increased our knowledge of hormonal receptor action. ERα rarely binds promoters and most estrogen receptor/chromatin interactions occur at distal enhancers (7), which are involved in chromatin loop structures to regulate gene expression (8). ERα/chromatin interactions require the functional involvement of other proteins, including FOXA1. FOXA1 is one of the key luminal-defining transcription factors (9, 10) and acts as pioneer factor for ERα by untangling chromatin structures, which enables ERα to bind its targets as shown in cell lines (11) and breast tumor samples (12). Other pioneer factors have been described for ERα function, including AP-2γ (13) and PBX1 (14), which function in synergy with FOXA1 in enabling ERα/chromatin interactions and subsequent activity. These reports tightly link ERα action with chromatin structure.
The epigenetic regulation of chromatin structure is a highly complex interplay between multiple histone modifiers and their downstream histone modifications, each with their intrinsically distinct patterns and functions (15). Two of the best-studied histone marks are the repressive trimethylation on lysine 27 of histone 3 (H3K27me3) (16) and the activating trimethylation on lysine 4 on histone 3 (H3K4me3) (17). H3K4me3 is found enriched at promoter regions (18, 19), whereas H3K27me3 can be found over large genomic regions spanning one or more epigenetically silenced genes (20, 21), which is directly regulated by the EZH2-driven polycomb repressive complex PRC2 (22).

To test for a possible interplay between differential ERα binding and epigenetic regulation of gene activity, we determined the genome-wide chromatin-binding patterns of ERα, H3K4me3, and H3K27me3 in primary breast tumor samples. Following relapse, patients were treated with an aromatase inhibitor for metastatic disease and time to progression (TTP) was correlated with altered ERα/H3K4me3/H3K27me3 binding profiles to identify distinct patterns that could hallmark aromatase inhibitor resistance.

MATERIALS AND METHODS

PATIENTS, TUMOR SAMPLES, AND PROCESSING

The Erasmus University Medical Center (EMC; Rotterdam, the Netherlands), the Netherlands Cancer Institute (Amsterdam, the Netherlands), and the Translational Cancer Research Unit (Saint Augustinus Hospital, Antwerpen, Belgium) participated in this study. A detailed description of the tumor samples has been described previously (23) and can be found in the Supplementary Methods.

In addition, previously described dataseries from patients with breast cancer, receiving neoadjuvant letrozole treatment (6) or tamoxifen for metastatic disease (24) were applied.

CHROMATIN IMMUNOPRECIPITATIONS

Chromatin immunoprecipitations (ChIP) were performed as described before (25) with minor adjustments. Firstly, as input material, tumor samples were cryosectioned (30× 30 µm sections) before further processing for ChIP-seq. For each ChIP, 10 µg of antibody was used, and 100 µL of Protein A magnetic beads (Invitrogen). ERα (SC-543; Santa Cruz), H3K4me3 (ab8580; Abcam), and H3K27me3 (07–449; Millipore) were used as antibodies. Primer sequences for quantitative PCR (qPCR) analyses are in Supplementary Table S5.

SOLEXA SEQUENCING AND ENRICHMENT ANALYSIS

ChIP DNA was amplified as described (25). Sequences were generated by the Illumina Hiseq 2000 genome analyzer (using 50 bp reads), and aligned to the Human Reference Genome (assembly hg19, February 2009). Enriched regions of the genome were identified by comparing the ChIP samples to mixed input using the MACS peak caller (26) version 1.3.7.1. Details on the number of reads obtained, the percentage of reads aligned, and the number of peaks called can be found in Supplementary Table S3. Bioinformatic analyses are described in the Supplementary Methods.

RNA ISOLATION AND MRNA EXPRESSION ANALYSIS

Total tumor RNA was isolated as described previously (27). mRNA quality was assessed by quantitative real-time PCR (qRT-PCR) and bioanalyzer. Amplification, labeling, and hybridization of samples to 44k mRNA oligonucleotide-arrays (Agilent Technologies) were performed as described (27). Samples were hybridized against a breast cancer reference pool consisting of RNA from 105 primary breast tumors. A detailed description of mRNA expression analyses and progression-free survival analyses can be found in the Supplementary Methods.

DATA ACCESS

All genomic data are deposited at the NCBI GEO, with accession numbers GSE40867 (ChIP-seq data) and GSE41994 (expression data).

RESULTS

GENOME-WIDE BINDING PATTERNS OF ERα, H3K4ME3, AND H3K27ME3 IN PRIMARY BREAST TUMOR SPECIMENS

Fresh frozen primary tumor specimens from a cohort of 84 patients with breast cancer were tested. These patients received aromatase inhibitor treatment of metastatic disease, i.e., anastrozole, letrozole, or exemestane (Supplementary Table S2). TTP was taken as an endpoint (Figure 1A). Poor outcome patients were defined as patients with a TTP < 12 months, whereas good outcome patients were defined as patients with a TTP > 24 months (Supplementary Tables S1 and S2). To determine the differential patterns in the ERα/chromatin–binding landscape and the epigenetic modifications H3K4me3 and H3K27me3 as well as their possible correlations with outcome after aromatase inhibitor treatment, five good outcome tumor samples and seven poor outcome tumors were randomly selected as "discovery set." Remaining samples from the entire cohort were used as "validation sets," as will be discussed later. The tumor cell percentage was consistently high (>70%), and all tumors were ERα and progesterone receptor (PR) positive, whereas negative for HER2. Other clinicopathologic parameters are shown in Supplementary Table S2.

For all 12 tumors from the discovery set, fresh frozen samples were cryosectioned, and chromatin was isolated for ChIP. For each tumor sample, the ChIP for ERα, H3K4me3, and H3K27me3 was performed and isolated DNA fragments were analyzed by high-throughput sequencing (ChIP-seq) (25). The number of aligned reads, unique reads, and number of peaks for each ChIP-seq sample are shown in Supplementary Table S3. Clear and distinct peaks were observed for each condition, as exemplified for one tumor sample (good outcome tumor #5; Figure 1B). In this example specimen, 13,575 binding events for ERα, 19,012 binding events for H3K4me3, and 33,661 binding events for H3K27me3 were found. Although the vast majority of binding events found from this tissue sample were unique among the markers, overlap was found between ERα and H3K4me3 as well as between H3K4me3 and H3K27me3 (Figure 1C; for all tumor samples, see Supplementary Figure S1). Even though the total numbers of binding events greatly varied among tumors, these relative distributions of the three ChIP conditions were consistently found for all tumor samples tested (Supplementary Figure S1). For the exemplified tumor sample, motif analysis was performed for each ChIP condition, Figure 1D. As expected, ERα–binding events were enriched for ESR1 motifs, but also for its designated pioneer factors FOXA1 (7) and TFAP2 (13). For H3K4me3 ChIP on this tumor sample, enriched motifs were found for the promoter-selective transcription factors GTF2I (28), ZIC1 (29), and E2F1:TFDP2 (30), whereas only motifs for the mitochondrial transcription factor A (TFAM) were observed for H3K27me3-bound regions.

For all tested tumor samples, example binding events for ERα, H3K4me3, and H3K27me3 are shown (Figure 1E), illustrating clear and high-quality data for all samples. For one tumor sample, no data could be generated for the H3K4me3 ChIP (poor outcome sample #2). Most ERα–binding events were found at distal enhancers and introns (Figure 1F),

consistent with previous reports in cell lines (7) and breast tumors (12). H3K4me3 was more markedly enriched at promoters, 3′-UTRs and exons, this in contrast to H3K27me3. These distributions were consistent in all analyzed tumors studied. The binding site distributions of all peaks related to the most proximal gene were found not to differ between good and poor outcome tumors.

FIGURE 1. Genome-wide DNA-binding patterns of ERα, H3K4me3, and H3K27me3 in primary human breast tumors.

A. Kaplan–Meier survival curve for the cohort of breast cancer patients who received aromatase inhibitors for metastatic disease. TTP < 12 months (red) is defined as poor outcome, whereas TTP > 24 months (green) is defined as good outcome.

B. Genome browser snapshot of ERα (red), H3K4me3 (blue), and H3K27me3 (green) ChIP-seq data from the same tumor sample. Genomic coordinates are indicated. Tag count is shown on the y-axis.

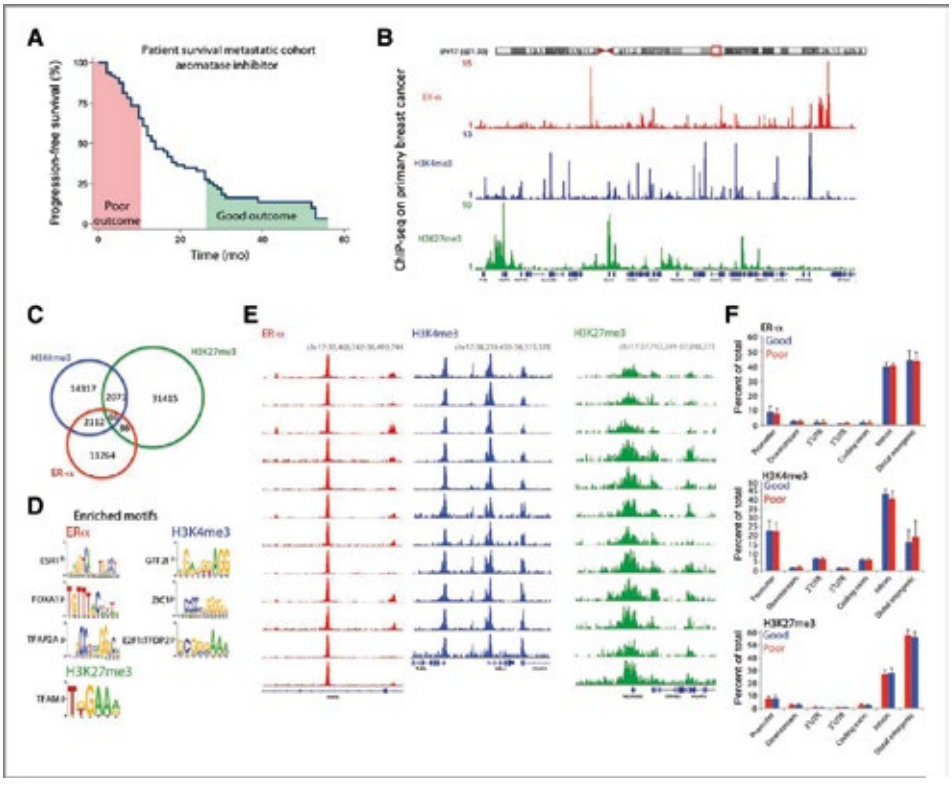
C. Venn diagram, illustrating shared and unique chromatinbinding events between ERa, H3K4me3, and H3K27me3 from the same human breast tumor specimen.

D. Enriched motifs for the ERα-, H3K4me3-, and H3K27me3–binding events, as highlighted in B and C.

E. Genome browser snapshot examples of binding events for ERα (red), H3K4me3 (blue), and H3K27me3 (green) for all the tested tumor samples.

F. Genomic distributions of binding events of ERα, H3K4me3, and H3K27me3 in poor (red) and good outcome (blue) tumors, illustrating the positioning of the binding events related to the most proximal gene.

Bars, SD values of all tested tumor samples.



DISTINCT GENOME-WIDE BINDING PATTERNS OF ERα, H3K4ME3, AND H3K27ME3 CORRELATE WITH PATIENT SURVIVAL AFTER AROMATASE INHIBITOR TREATMENT

Next, we aimed to determine whether the chromatin-binding patterns of ERα-, H3K4me3-, and H3K27me3–binding events would deviate between patients with a good versus a poor outcome upon aromatase inhibitor treatment. This was achieved through differential binding analysis (DBA) (12, 31), directly comparing good outcome and poor outcome tumor ChIP-seq data. DBA normalizes the sequencing data for each run over the effective library size (reads in peaks) after input background subtraction, and determines relative enrichment of raw sequence reads over two distinct subgroups of tumor samples, in this case good versus poor outcome. Called peaks that were found in at least two tumor samples were considered for DBA to minimize noise, resulting in 14,232 peaks for ERα, 22,587 peaks for H3K4me3, and 35,602 peaks for H3K27me3 (Supplementary Figure S2). On the basis of these peaks, the raw read counts for all tumor samples were checked for differential binding intensities between the two patient subgroups, resulting in lists of 222 (ERα), 66 (H3K4me3), and 351 (H3K27me3) peaks, with a false discovery rate of < 0.1 (Figure 2A). Tumors from patients with the same outcome clustered together for ERα and H3K27me3 and, although less pronounced, for the H3K4me3 signals (Figure 2B). This class separability was also observed in a principal component analysis (Supplementary Figure S3). The read count and peak caller score did not bias patient stratification (Supplementary Figure S4).

FIGURE 2. (on the right) Distinct chromatin-binding patterns of ERα and H3K27me3 in tumor samples with differential aromatase inhibitor response.

A. Example genomic regions with differential ERα (red), H3K4me3 (blue), and H3K27me3 (green) binding events. Genomic coordinates are indicated. Tag count is shown for each position.

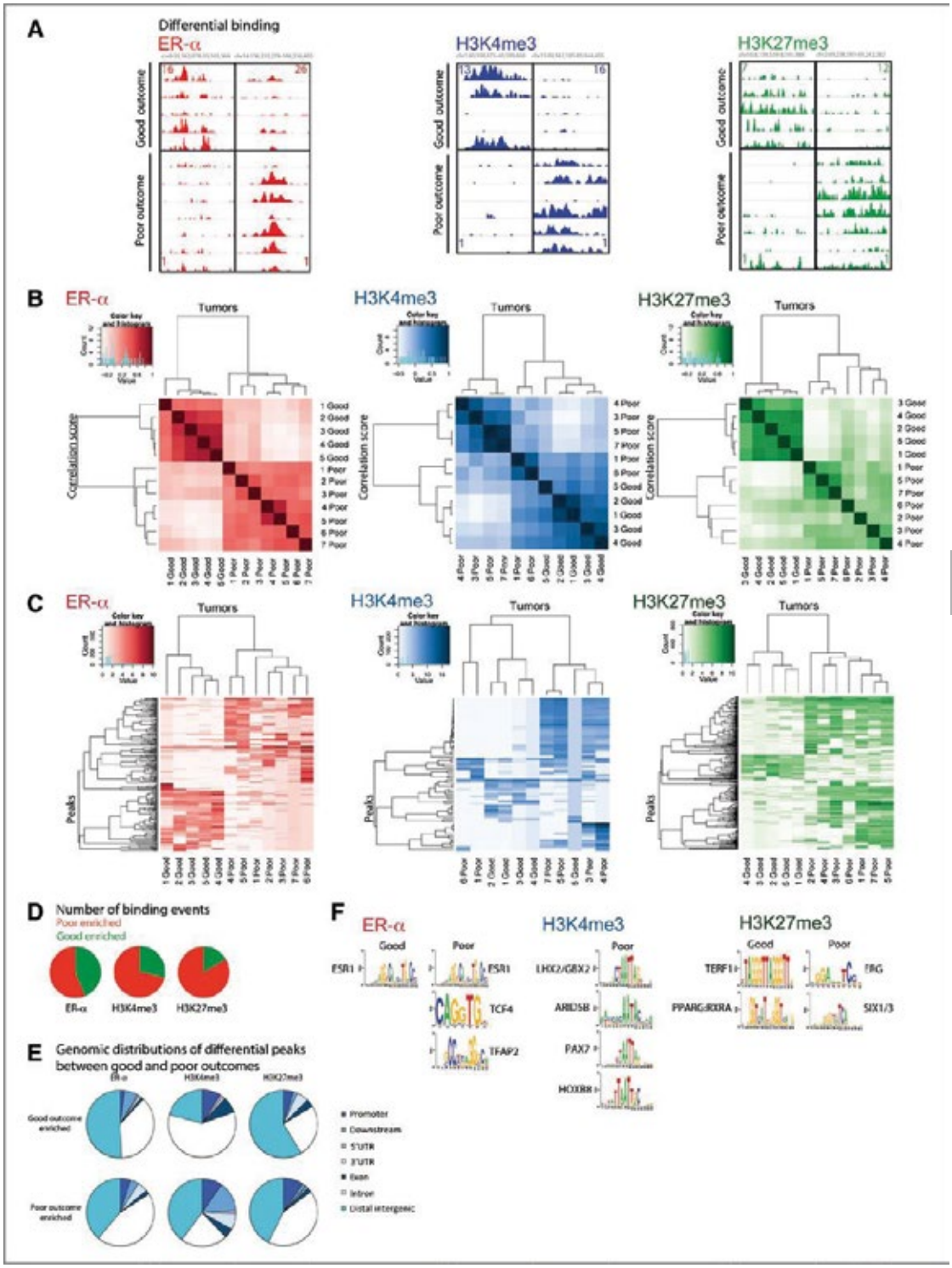
B. Cross-correlation analysis for ERα (red), H3K4me3 (blue), and H3K27me3 (green) on the basis of the differential-bound profiles. Heatmap intensities and counts are depicted in the top left corner for each factor.

C. Heatmap visualization of the ERα (red), H3K4me3 (blue), and H3K27me3 (green) peak intensities that were differentially enriched in good and poor outcome tumors. Heatmap intensities and counts are depicted in the top left corner for each factor.

D. Pie chart depicting the relative number of peaks that were differentially enriched in good and poor outcome tumors.

E. Genomic locations of the differentially enriched peaks of ERα, H3K4me3, and H3K27me3 in good and poor outcome, related to the most proximal genes.

F. Top motif enrichment for the unique binding events in good and poor outcome tumors for all three markers. No enriched motifs were found for the H3K4me3 peaks that were only observed in the good outcome patients.


A
Differential binding
ER-α
H3K4me3
H3K27me3
Good outcome
Poor outcome
B
ER-α
H3K4me3
H3K27me3
Tumors
Correlation score
C
ER-α
H3K4me3
H3K27me3
Tumors
Peaks
D
Number of binding events
Poor enriched
Good enriched
ER-α
H3K4me3
H3K27me3
E
Genomic distributions of differential peaks between good and poor outcomes
Good outcome enriched
Poor outcome enriched
Promoter
Downstream
5'UTR
3'UTR
Exon
Intron
Distal intergenic
F
ER-α
H3K4me3
H3K27me3
Good
Poor
ESR1
TCF4
TFAP2
LHX2/GBX2
ARID5B
PAX7
HOXB8
TERF1
PPARG:RXRA
TBG
SIX1/3

Peak regions that were significantly differentially enriched between the poor and good outcome patients are shown in a heatmap for all three conditions (Figure 2C), with a clear distinction for ERα and H3K27me3, whereas case mixing was observed for H3K4me3 signals. The number of poor outcome peaks greatly outweighs the number of good outcome events for both histone marks, but not for ERα (Figure 2D). A list of all differentially bound regions between good and poor outcome for ERα, H3K4me3, and H3K27me3 is shown in Supplementary Table S4.

The binding events that were differentially enriched between the two patient subgroups were analyzed for their localization relative to the most proximal genes (Figure 2E). No clear differences in genomic locations of the ERα peaks were observed between good and poor outcome patients. H3K4me3 poor outcome peaks were found enriched at 3'-UTR regions and downstream thereof, whereas the 3'-UTR signals for H3K27me3-binding events were selectively lost in poor outcome tumor samples.

The ERα-, H3K4me3-, and H3K27me3–binding events were analyzed for DNA motifs differentially enriched in the good and poor outcome patient subgroups (Figure 2F). Although only ESR1 motifs were found in good outcome patients, TFAP2A and TCF4 motifs were enriched next to ESR1 for the poor outcome patients. For H3K27me3, good outcome–enriched binding events were enriched for TERF1 and PPARG:RXRA motifs, whereas for poor outcome–enriched binding events, motifs were found for ERG and SIX1/SIX3. No distinct enriched motifs were found for the good outcome–enriched binding event for H3K4me3, but the poor outcome sites were statistically enriched for LHX2/GBX2, ARID5B, PAX7, and HOXB8 motifs.

MUTUAL EXCLUSIVITY OF DIFFERENTIALLY ENRICHED CHROMATIN-BINDING EVENTS BETWEEN THE PATIENT SUBGROUPS

A large variation was found in the number (Supplementary Table S2) and overlap of chromatin-binding events between samples as exemplified for three specimens (Figure 3A; for all samples Supplementary Figure S2). This is consistent with a previous report that studied ERα ChIP-seq on breast tumor specimens (12). Because ERα/chromatin interactions are dictated by and have a facilitating effect on histone accessibility (11), we determined if altered ERα–binding patterns are accompanied by changes in the epigenetic profile of H3K4me3 and H3K27me3. When ERα–binding events observed in the poor outcome tumor sample were absent in the good outcome tumor, these alterations are not accompanied by a loss or gain of proximal histone marks, as exemplified in Figure 3B. In line with these data, differences of either histone mark between good and poor outcome patients were virtually mutually exclusive and not shared with changed ERα–binding patterns (Figure 3C). Consequently, a limited overlap was observed for the genes that were proximal to the altered ERα-, H3K4me3-, and H3K27me3–binding events (Figure 3D). The altered binding events were mapped over all chromosomes and no clear bias towards distinct regions or chromosomes was observed (Figure 3E).

FIGURE 3. Mutual exclusivity of altered binding events.

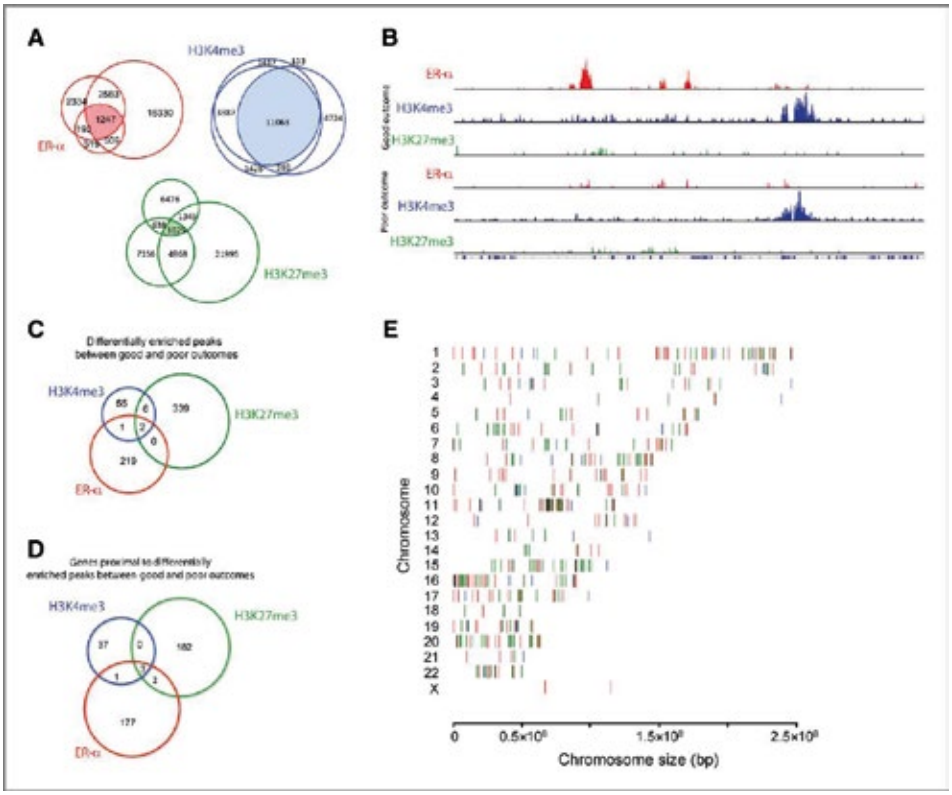
A. Venn diagram, showing shared and unique binding events of ERα (red), H3K4me3 (blue), and H3K27me3 (green) from three example tumor samples. ERα and H3K27me3–binding patterns are highly heterogeneous between tumors, in contrast to H3K4me3-binding events.

B. Genome browser snapshot from a good outcome (top) and a poor outcome (bottom) tumor sample. Even though the ERα–binding sites between the two tumor samples were altered, no change was found for the present H3K4me3 (blue) and absent H3K27me3 (green) signals.

C. Venn diagram, showing the shared and unique differentially bound binding events for ERα, H3K4me3, and H3K27me3, comparing good and poor outcome patients.

D. As in C, but now analyzing the genes proximal to the altered binding events. All peaks within a gene body or 20 kb upstream from the transcription start site (TSS) were considered as proximal.

E. Visualization of the genome-wide distribution of the altered binding events of ERα (red), H3K4me3 (blue), and H3K27me3 (green) depicted over all chromosomes.



VALIDATION, INTEGRATION WITH GENE EXPRESSION, AND CLINICAL OUTCOME

Next, differential enriched binding patterns between good and poor outcome patients were validated in an independent group of 10 patients (4 good outcome and 6 poor outcome) using qPCR, Figure 4A (for clinicopathologic parameters, see Supplementary Table S2). For each of the ChIP conditions, four to six primer pairs were designed, detecting randomly picked regions enriched in the good or poor outcome patients from the discovery set. ChIP efficiency can vary among samples due to tumor cell percentage, expression levels, and experimental variations, making inter-sample normalization an essential step in the DBA for the ChIP-seq pipeline. To implement inter-sample normalization in qPCR, the ratios of average good outcome over poor outcome–enriched binding site intensities were calculated. For ERα and H3K27me3, qPCR ratio separated poor outcome patients from good outcome patients, whereas differential enrichment could not be confirmed for H3K4me3.

After qPCR validation, all ChIP-seq–identified differential binding patterns were coupled to genes, based on proximity (gene body plus 20 kb upstream from the transcription start-site), enriching for ERα–binding sites involved in gene regulation (8). For the good outcome binding sites, this resulted in 84 (ERα), 19 (H3K4me3), and 29 (H3K27me3) genes. For poor outcome sites, 99 (ERα), 22 (H3K4me3), and 158 (H3K27me3) genes were found (Supplementary Table S6).

Gene Ontology (GO) analyses were performed on the proximal gene sets to identify plausible functional regulatory networks (Supplementary Table S7). Genes between the different classifiers only marginally overlapped (Figure 3D), whereas pathways between ERα, H3K4me3, and H3K27me3-based classifiers showed no overlap. The ERα–based classifier was enriched for metabolic processes, whereas nucleoside transport, chemotaxis, and angiogenesis were enriched GO terms for H3K4me3. For the H3K27me3 classifier, developmental processes were strongly enriched. Comparing our data with pathways identified in mutational analysis of aromatase inhibitor–treated patients with breast cancer (4) showed shared processes involved in cell adhesion, cell cycle, chemotaxis, developmental processes, immune responses, metabolism, signal transduction, and transcriptional regulation (Supplementary Table S8).

Next, array-based mRNA expression levels of all proximal genes for ERα, H3K4me3, and H3K27me3 sites were tested for a correlation with time to progression (TTP) in a second independent set of 72 tumors (validation set), of aromatase inhibitor–treated patients with metastatic disease (Figure 4B, clinicopathologic parameters Supplementary Table S2). Expression of proximal genes for differential ERα (183 genes), H3K4me3 (41 genes), and H3K27me3 (187 genes) binding was used to classify tumors as “poor” and “good outcome.” Classifications for altered ERα (P = 0.0016; HR, 2.42; 95% CI, 1.40–4.19) and H3K27me3 (P = 0.0001; HR, 3.19; 95% CI, 1.83–5.57) binding events correlated with a differential TTP, in contrast to H3K4me3 (P = 0.386; HR, 1.38; 95% CI, 0.76–2.49).

Previously reported classifiers, PAM50 (P = 0.0093; HR, 2.11; 95% CI, 1.20–3.71) (32), Oncotype DX (P = 0.0256; HR, 1.87; 95% CI, 1.08–3.23) (33), and the TAM78 Rotterdam classifier (P = 0.0151; HR, 1.98; 95% CI, 1.14–3.42) (34), classified patients in this cohort, performing equally well as the ERα–proximal gene classifier (Figure 4E). The H3K27me3-proximal gene classifier showed a higher HR, even though the 95% CI did overlap (Figure 4E). ERα and H3K27me3 ChIP-seq–based classifiers remained significant after multivariate correction analyses (Supplementary Table S9). The classifier genes are listed in Supplementary Table S6. As a second expression-based validation, a cohort of neoadjuvant letrozole treatment patients (N = 54) was used, analyzing samples before and after treatment, where patients were stratified in responsive and non-responsive groups (Figure 4C) (5, 6). PAM50, Oncotype DX, and TAM78 as well as our ChIP-seq classifiers successfully identified patients with differential outcome.

ERα can affect gene regulation by long-range genomic chromatin-loop interactions, as shown by ERα ChIA-PET (8). Therefore, these published long-range interactions were also considered in our study. In addition, binding sites were analyzed for any transcription factor enrichment, DNAse hypersensitivity, and H3K27Ac, representing active enhancers (35), using ENCODE datasets (Supplementary Table S10). Genes were selected that either had a binding event at a promoter region, or chromatin looping toward the promoter was found, generating three separate lists of 92 (ERα), 38 (H3K4me3), and 150 (H3K27me3) genes. Overlap between the "proximity-based" and "ChIA-PET–based" gene sets was high for H3K4me3 (76%) and H3K27me3 (78%), but considerably lower for ERα (49%; Supplementary Figure S5). Analogous to our initial classifiers, ChIA-PET–based classifiers for ERα (P = 0.0041; HR, 2.45; 95% CI, 1.41–4.26) and H3K27me3 (P = 0.0002; HR, 3.02; 95% CI, 1.73–5.27) could classify patients, in contrast to H3K4me3 (P = 0.606; HR, 1.30; 95% CI, 0.71–2.37; Supplementary Figure S6A and Supplementary Table S9). Also for this "ChIA-PET–based" classifier, samples from the neoadjuvant treated letrozole cohort were analyzed (6), identifying patients with differential response (Supplementary Figure S6B). Conserved genes between both gene classifiers performed equally as the separate classifications (ERα: P = 0.05; HR, 1.96, 95% CI, 1.10–3.49; H3K27me3: P = 0.0001; HR, 3.68, CI 95%, 2.09–6.50; H3K4me3: P = 0.3944; HR, 1.30, 95% CI, 0.711–2.37; Supplementary Figure S6C and S6D).

A third cohort of patients was analyzed, receiving first-line tamoxifen for metastatic disease instead of aromatase inhibitors (Figure 4D) (27). The metastatic setup of this cohort enabled a direct comparison between the patient sets. No significant correlation with TTP after tamoxifen was found for the H3K4me3 and H3K27me3 ChIP-seq–based classifiers (H3K27me3: P = 0.0838, HR, 1.53, 95% CI, 0.95–2.47; and H3K4me3: P = 0.0602, HR, 1.556, 95% CI, 0.98–2.47). However, significance was reached for the ERα–based classifier (P = 0.0426, HR, 1.63, 95% CI, 1.02–2.63). These data indicate that while the ERα ChIP-seq–based classifier may identify breast cancer patients with a poor outcome, irrespective of the type of endocrine treatment, the H3K27me3 classification suggests aromatase inhibitor–treatment selectivity in patients with metastatic breast cancer (Figure 4E).

FIGURE 4. ChIP-qPCR validation and correlation with survival and treatment selectivity.

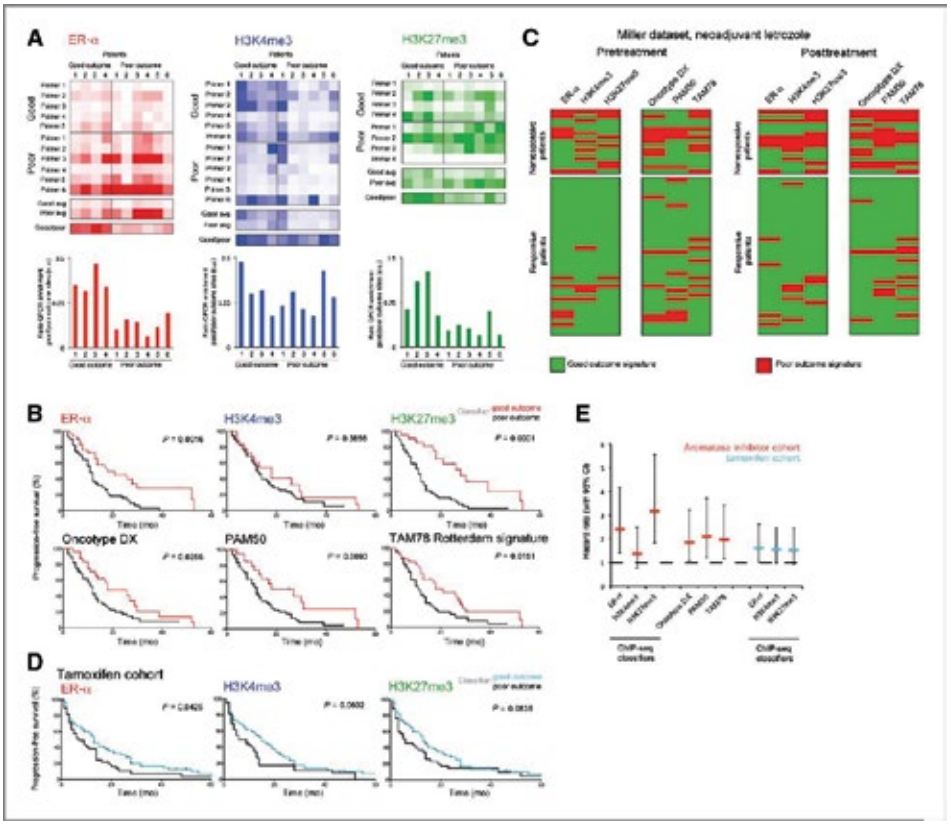
A. Heatmap, illustrating qPCR-ChIP–based enrichment of regions enriched for patients with a good or poor outcome after aromatase inhibitor treatment, normalized over negative control CCND1 primers. Average signals for the good outcome and poor outcome sites were calculated and ratio was determined, as also visualized in a bar plot (bottom). Separate primer sets were used for the altered ERα (red), H3K4me3 (blue), and H3K27me3 (green) binding events.

B. Kaplan–Meier survival curves of aromatase inhibitor–treated patients, using the proximal genes from the differentially enriched binding patterns of ERα, H3K4me3, or H3K27me3, as well as the established Oncotype DX, PAM50, and TAM78 as classifiers. TTP is shown with time expressed in months.

C. Heatmap visualization of patient classification. Gene expression data from the Miller dataset were applied, where 54 patients received neoadjuvant letrozole treatment. Patients were stratified in responders and nonresponders, and pre- and posttreated samples from the same patients were separately analyzed. Adherence to the gene classifier is visualized in a heatmap, where green indicates a good outcome signature and red a poor outcome signature.

D. Identical analyses as depicted in B, but now applying the aromatase inhibitor ChIP-seq–based classifiers on a cohort of breast cancer patients who received tamoxifen for metastatic disease.

E. Hazard rates (HR), including 95% CI values, for the ChIP-seq–based classifiers (ERα, H3K4me3, or H3K27me3) as compared with established Oncotype DX, PAM50, and TAM78 classifiers. Both the aromatase inhibitor cohort (red) and tamoxifen cohort (blue) of patients treated for metastatic disease are shown.



DISCUSSION

Estrogen receptor genomics has greatly increased our knowledge of hormone receptor functioning in breast cancer cell lines and tumors. The number of ERα/chromatin–binding events greatly exceeds the amount of estradiol-responsive genes (11, 36), suggesting a high level of complexity in ERα–mediated gene regulation (8). ERα/chromatin–binding events have been found to correlate with survival of patients with breast cancer (12), but survival in this heterogeneous study also correlated with traditional pathologic parameters, including PR and HER2, potentially hampering further clinical interpretation of the data. Therefore, we selected ERα+, PR+, and HER2− tumors from a homogeneous cohort of breast cancer patients whose metastasis were all treated with aromatase inhibitors.

Altered ERα–binding patterns between patient groups were not accompanied with altered epigenetic profiles of H3K4me3 and H3K27me3. Our data suggest that any dynamic behavior of ERα uses the accessible regions in the genome that are imprinted and readily accessible in a static epigenetic landscape. Still, H3K27me3-binding events enable the identification of patients with a poor outcome after aromatase inhibitor treatment, in contrast to H3K4me3. The presented data suggest that aromatase inhibitor resistance is accompanied by a specific gain of polycomb-mediated gene repression at distinct sites. Moreover, it suggests an ERα–independent mechanism of therapy resistance in ERα–positive tumors. Our data could be validated in two other cohorts of breast cancer patients, using two different technologic approaches, namely ChIP-qPCR and gene expression analysis.

Because genomic patterns of ERα and H3K27me3 were indicative for patient survival, the enriched motifs may provide clues for transcription factors involved in treatment outcome. For ERα, TFAP2 and TCF4 motifs were selectively enriched for the poor outcome patients. AP-2 can directly guide ERα/chromatin interactions (13), promotes breast cancer cell proliferation (37), and correlates with poor outcome (38). TCF4 enhances breast cancer cell invasion (39) and binds ERα (40), providing a level of cross-control between estradiol and wnt pathways (40).

For H3K27me3, “good outcome” sites were enriched for TERF1 and PPARG:RXRA motifs. TERF1 is a component of the telomere nucleoprotein complex. SNPs in TERF1 have been tested for breast cancer susceptibility and prognosis, but no correlations were found (41). PPARG:RXRA ligands can trigger breast cancer cell apoptosis (42) and PPARγ activation blocks breast cancer cell invasion (43) and induces terminal cell differentiation (44). For poor outcome–enriched H3K27me3 regions, motifs were found for ERG and SIX1/SIX3. ERG and ERα mutually repress each other’s activities (45). SIX1 is expressed in breast cancer, stimulating tumor cell proliferation (46), inducing genomic instability and malignant transformation (47), correlating with poor prognosis (48). Collectively, these data highlight possible transcriptional mechanisms that may form the basis for aromatase inhibitor response.

The ERα ChIP-seq classifier was applicable for aromatase inhibitor- and tamoxifen–treated patients with breast cancer with metastatic disease. Because aromatase inhibitors and tamoxifen both affect the functionality of ERα, the genomic downstream signatures for treatment outcome could overlap as well. Because ERα is targeted by these endocrine agents, the ChIP-seq approach could aid in removing noise from expression analyses to exclusively monitor genes that are directly affected by this hormone receptor.

Testing the aromatase inhibitor ChIP-seq classifiers in tamoxifen-treated tumors showed no significant difference in TTP for H3K27me3. Aromatase inhibitor–treated patients cohorts are relatively rare, and our cohort is on metastatic disease. To compare between endocrine treatments, data from a metastatic cohort of tamoxifen-treated patients were used. Performing these ERα ChIP-seq experiments and epigenetic assessments in the adjuvant setting would enable a direct comparison with any of the large (adjuvant-treated) breast cancer patient cohorts (34, 49, 50) for more extensive *in silico* validations. Tumor-intrinsic plasticity of ERα and H3K27me3 can be a hallmark of endocrine therapy resistance in breast cancer and may ultimately be applicable to guide endocrine treatment selection for patients with breast cancer.

REFERENCES

1. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer. 2010;127(12):2893-917.
2. Baselga J, Campone M, Piccart M, Burris HA, 3rd, Rugo HS, Sahmoud T, et al. Everolimus in postmenopausal hormone-receptor-positive advanced breast cancer. N Engl J Med. 2012;366(6):520-9.
3. Bertelli G. Sequencing of aromatase inhibitors. Br J Cancer. 2005;93 Suppl 1:S6-9.
4. Ellis MJ, Ding L, Shen D, Luo J, Suman VJ, Wallis JW, et al. Whole-genome analysis informs breast cancer response to aromatase inhibition. Nature. 2012;486(7403):353-60.
5. Miller WR, Larionov A. Changes in expression of oestrogen regulated and proliferation genes with neoadjuvant treatment highlight heterogeneity of clinical resistance to the aromatase inhibitor, letrozole. Breast Cancer Res. 2010;12(4):R52.
6. Miller WR, Larionov A, Renshaw L, Anderson TJ, Walker JR, Krause A, et al. Gene expression profiles differentiating between breast cancers clinically responsive or resistant to letrozole. J Clin Oncol. 2009;27(9):1382-
7. Carroll JS, Liu XS, Brodsky AS, Li W, Meyer CA, Szary AJ, et al. Chromosome-wide mapping of estrogen receptor binding reveals long-range regulation requiring the forkhead protein FoxA1. Cell. 2005;122(1):33-43.
8. Fullwood MJ, Liu MH, Pan YF, Liu J, Xu H, Mohamed YB, et al. An oestrogen-receptor-alpha-bound human chromatin interactome. Nature. 2009;462(7269):58-64.
9. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. Nature. 2000;406(6797):747-52.
10. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A. 2001;98(19):10869-74.
11. Hurtado A, Holmes KA, Ross-Innes CS, Schmidt D, Carroll JS. FOXA1 is a key determinant of estrogen receptor function and endocrine response. Nat Genet. 2011;43(1):27-33.
12. Ross-Innes CS, Stark R, Teschendorff AE, Holmes KA, Ali HR, Dunning MJ, et al. Differential oestrogen receptor binding is associated with clinical outcome in breast cancer. Nature. 2012;481(7381):389-93.
13. Tan SK, Lin ZH, Chang CW, Varang V, Chng KR, Pan YF, et al. AP-2gamma regulates oestrogen receptor-mediated long-range chromatin interaction and gene transcription. EMBO J. 2011;30(13):2569-81.
14. Magnani L, Ballantyne EB, Zhang X, Lupien M. PBX1 genomic pioneer function drives ERalpha signaling underlying progression in breast cancer. PLoS Genet. 2011;7(11):e1002368.
15. Ernst J, Kheradpour P, Mikkelsen TS, Shoresh N, Ward LD, Epstein CB, et al. Mapping and analysis of chromatin state dynamics in nine human cell types. Nature. 2011;473(7345):43-9.
16. Agger K, Cloos PA, Christensen J, Pasini D, Rose S, Rappsilber J, et al. UTX and JMJD3 are histone H3K27 demethylases involved in HOX gene regulation and development. Nature. 2007;449(7163):731-4.
17. Bernstein BE, Humphrey EL, Erlich RL, Schneider R, Bouman P, Liu JS, et al. Methylation

of histone H3 Lys 4 in coding regions of active genes. Proc Natl Acad Sci U S A. 2002;99(13):8695-700.
18. Guenther MG, Levine SS, Boyer LA, Jaenisch R, Young RA. A chromatin landmark and transcription initiation at most promoters in human cells. Cell. 2007;130(1):77-88.
19. Kirmizis A, Santos-Rosa H, Penkett CJ, Singer MA, Vermeulen M, Mann M, et al. Arginine methylation at histone H3R2 controls deposition of H3K4 trimethylation. Nature. 2007;449(7164):928-32.
20. Mikkelsen TS, Ku M, Jaffe DB, Issac B, Lieberman E, Giannoukos G, et al. Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. Nature. 2007;448(7153):553-60.
21. Barski A, Cuddapah S, Cui K, Roh TY, Schones DE, Wang Z, et al. High-resolution profiling of histone methylations in the human genome. Cell. 2007;129(4):823-37.
22. Hansen KH, Bracken AP, Pasini D, Dietrich N, Gehani SS, Monrad A, et al. A model for transmission of the H3K27me3 epigenetic mark. Nat Cell Biol. 2008;10(11):1291-300.
23. Ramirez-Ardila DE, Helmijr JC, Look MP, Lurkin I, Ruigrok-Ritstier K, van Laere S, et al. Hotspot mutations in PIK3CA associate with first-line treatment outcome for aromatase inhibitors but not for tamoxifen. Breast Cancer Res Treat. 2013;139(1):39-49.
24. Kok M, Koornstra RH, Mook S, Hauptmann M, Fles R, Jansen MP, et al. Additional value of the 70-gene signature and levels of ER and PR for the prediction of outcome in tamoxifen-treated ER-positive breast cancer. Breast. 2012;21(6):769-78.
25. Schmidt D, Wilson MD, Spyrou C, Brown GD, Hadfield J, Odom DT. ChIP-seq: using high-throughput sequencing to discover protein-DNA interactions. Methods. 2009;48(3):240-8.
26. Zhang Y, Liu T, Meyer CA, Eeckhoute J, Johnson DS, Bernstein BE, et al. Model-based analysis of ChIP-Seq (MACS). Genome Biol. 2008;9(9):R137.
27. Kok M, Linn SC, Van Laar RK, Jansen MP, van den Berg TM, Delahaye LJ, et al. Comparison of gene expression profiles predicting progression in breast cancer patients treated with tamoxifen. Breast Cancer Res Treat. 2009;113(2):275-83.
28. Makeyev AV, Enkhmandakh B, Hong SH, Joshi P, Shin DG, Bayarsaihan D. Diversity and complexity in chromatin recognition by TFII-I transcription factors in pluripotent embryonic stem cells and embryonic tissues. PLoS One. 2012;7(9):e44443.
29. Salero E, Perez-Sen R, Aruga J, Gimenez C, Zafra F. Transcription factors Zic1 and Zic2 bind and transactivate the apolipoprotein E gene promoter. J Biol Chem. 2001;276(3):1881-8.
30. Liu W, Tanasa B, Tyurina OV, Zhou TY, Gassmann R, Liu WT, et al. PHF8 mediates histone H4 lysine 20 demethylation events involved in cell cycle progression. Nature. 2010;466(7305):508-12.
31. Sharma NL, Massie CE, Ramos-Montoya A, Zecchini V, Scott HE, Lamb AD, et al. The androgen receptor induces a distinct transcriptional program in castration-resistant prostate cancer in man. Cancer Cell. 2013;23(1):35-47.
32. Parker JS, Mullins M, Cheang MC, Leung S, Voduc D, Vickery T, et al. Supervised risk predictor of breast cancer based on intrinsic subtypes. J Clin Oncol. 2009;27(8):1160-7.
33. Cobleigh MA, Tabesh B, Bitterman P, Baker J, Cronin M, Liu ML, et al. Tumor gene expression and prognosis in breast cancer patients with 10 or more positive lymph nodes. Clin Cancer Res. 2005;11(24 Pt 1):8623-31.

34. Wang Y, Klijn JG, Zhang Y, Sieuwerts AM, Look MP, Yang F, et al. Gene-expression profiles to predict distant metastasis of lymph-node-negative primary breast cancer. Lancet. 2005;365(9460):671-9.
35. Creyghton MP, Cheng AW, Welstead GG, Kooistra T, Carey BW, Steine EJ, et al. Histone H3K27ac separates active from poised enhancers and predicts developmental state. Proc Natl Acad Sci U S A. 2010;107(50):21931-6.
36. Zwart W, Theodorou V, Kok M, Canisius S, Linn S, Carroll JS. Oestrogen receptor-co-factor-chromatin specificity in the transcriptional regulation of breast cancer. EMBO J. 2011;30(23):4764-76.
37. Williams CM, Scibetta AG, Friedrich JK, Canosa M, Berlato C, Moss CH, et al. AP-2gamma promotes proliferation in breast tumour cells by direct repression of the CDKN1A gene. EMBO J. 2009;28(22):3591-601.
38. Gee JM, Eloranta JJ, Ibbitt JC, Robertson JF, Ellis IO, Williams T, et al. Overexpression of TFAP2C in invasive breast cancer correlates with a poorer response to anti-hormone therapy and reduced patient survival. J Pathol. 2009;217(1):32-41.
39. Ravindranath A, Yuen HF, Chan KK, Grills C, Fennell DA, Lappin TR, et al. Wnt-beta-catenin-Tcf-4 signalling-modulated invasiveness is dependent on osteopontin expression in breast cancer. Br J Cancer. 2011;105(4):542-51.
40. El-Tanani M, Fernig DG, Barraclough R, Green C, Rudland P. Differential modulation of transcriptional activity of estrogen receptors by direct protein-protein interactions with the T cell factor family of transcription factors. J Biol Chem. 2001;276(45):41675-82.
41. Varadi V, Brendle A, Brandt A, Johansson R, Enquist K, Henriksson R, et al. Polymorphisms in telomere-associated genes, breast cancer susceptibility and prognosis. Eur J Cancer. 2009;45(17):3008-16.
42. Bonofiglio D, Cione E, Qi H, Pingitore A, Perri M, Catalano S, et al. Combined low doses of PPARgamma and RXR ligands trigger an intrinsic apoptotic pathway in human breast cancer cells. Am J Pathol. 2009;175(3):1270-80.
43. Liu H, Zang C, Fenner MH, Possinger K, Elstner E. PPARgamma ligands and ATRA inhibit the invasion of human breast cancer cells in vitro. Breast Cancer Res Treat. 2003;79(1):63-74.
44. Mueller E, Sarraf P, Tontonoz P, Evans RM, Martin KJ, Zhang M, et al. Terminal differentiation of human breast cancer through PPAR gamma. Mol Cell. 1998;1(3):465-70.
45. Vlaeminck-Guillem V, Vanacker JM, Verger A, Tomavo N, Stehelin D, Laudet V, et al. Mutual repression of transcriptional activation between the ETS-related factor ERG and estrogen receptor. Oncogene. 2003;22(50):8072-84.
46. Coletta RD, Christensen K, Reichenberger KJ, Lamb J, Micomonaco D, Huang L, et al. The Six1 homeoprotein stimulates tumorigenesis by reactivation of cyclin A1. Proc Natl Acad Sci U S A. 2004;101(17):6478-83.
47. Coletta RD, Christensen KL, Micalizzi DS, Jedlicka P, Varella-Garcia M, Ford HL. Six1 overexpression in mammary cells induces genomic instability and is sufficient for malignant transformation. Cancer Res. 2008;68(7):2204-13.

48. Iwanaga R, Wang CA, Micalizzi DS, Harrell JC, Jedlicka P, Sartorius CA, et al. Expression of Six1 in luminal breast cancers predicts poor prognosis and promotes increases in tumor initiating cells by activation of extracellular signal-regulated kinase and transforming growth factor-beta signaling pathways. Breast Cancer Res. 2012;14(4):R100.
49. Loi S, Haibe-Kains B, Desmedt C, Lallemand F, Tutt AM, Gillet C, et al. Definition of clinically distinct molecular subtypes in estrogen receptor-positive breast carcinomas through genomic grade. J Clin Oncol. 2007;25(10):1239-46.
50. Buffa FM, Camps C, Winchester L, Snell CE, Gee HE, Sheldon H, et al. microRNA-associated progression pathways and potential therapeutic targets identified by integrated mRNA and microRNA expression profiling in breast cancer. Cancer Res. 2011;71(17):5635-45.

SUPPLEMENTARY MATERIAL

https://drive.google.com/open?id=0B_eW3FoE0HzYdEJNZHlUcWx6ODg

CHAPTER 6

An 8-gene mRNA expression profile in circulating tumor cells predicts response to aromatase inhibitors in metastatic breast cancer patients

E.A. Reijm, A.M. Sieuwerts, M. Smid, J. Bolt-de Vries, B. Mostert, W. Onstenk, D. Peeters, L.Y. Dirix, C.M. Seynaeve, A. Jager, F.E. de Jongh, P. Hamberg, A. van Galen, J. Kraan, M.P.H.M. Jansen, J.W. Gratama, J.A. Foekens, J.W.M. Martens, E.M.J.J. Berns, and S. Sleijfer

BMC Cancer 2016; 16:123

ABSTRACT

BACKGROUND

Molecular characterization of circulating tumor cells (CTC) is promising for personalized medicine. We aimed to identify a CTC gene expression profile predicting outcome to first-line aromatase inhibitors in metastatic breast cancer (MBC) patients.

METHODS

CTCs were isolated from 78 MBC patients before treatment start. mRNA expression levels of 96 genes were measured by quantitative reverse transcriptase polymerase chain reaction. After applying predefined exclusion criteria based on lack of sufficient RNA quality and/or quantity, the data from 45 patients were used to construct a gene expression profile to predict poor responding patients, defined as disease progression or death <9 months, by a leave-one-out cross validation.

RESULTS

Of the 45 patients, 19 were clinically classified as poor responders. To identify them, the 75% most variable genes were used to select genes differentially expressed between good and poor responders. An 8-gene CTC predictor was significantly associated with outcome (Hazard Ratio [HR] 4.40, 95 % Confidence Interval [CI]: 2.17–8.92, $P < 0.001$). This predictor identified poor responding patients with a sensitivity of 63% and a positive predictive value of 75%, while good responding patients were correctly predicted in 85% of the cases. In multivariate Cox regression analysis, including CTC count at baseline, the 8-gene CTC predictor was the only factor independently associated with outcome (HR 4.59 [95% CI: 2.11–9.56], $P < 0.001$). This 8-gene signature was not associated with outcome in a group of 71 MBC patients treated with systemic treatments other than AI.

CONCLUSIONS

An 8-gene CTC predictor was identified which discriminates good and poor outcome to first-line aromatase inhibitors in MBC patients. Although results need to be validated, this study underscores the potential of molecular characterization of CTCs.

INTRODUCTION

Metastatic breast cancer (MBC) is a highly heterogeneous disease leading to an urgent need for a more personalized treatment approach. For those patients with estrogen receptor (ER)-expressing tumors, endocrine therapy is the mainstay of treatment. Although many patients greatly benefit from such endocrine therapies, approximately 30% of the MBC patients never respond while virtually all initial responders eventually relapse and develop progressive disease. Numerous factors accounting for resistance to endocrine treatment have been revealed, including loss of ER expression (1-3), overexpression of the HER2 receptor (4), hyperactivation of the phosphatidylinositol 3-kinase (PI3K) pathway (5), and overexpression of Enhancer of Zeste Homolog 2 (EZH2) (6). Determination of these factors in tumor tissue may therefore contribute to a more personalized treatment approach of individual patients.

Predictive factors contributing to treatment decision making are nowadays most commonly identified in the primary tumors. However, heterogeneity in molecular characteristics between primary tumor and metastases, including clinically relevant factors, is increasingly recognized. For example, differences in ER expression between primary tumor and metastases occur in approximately 20% of the patients leading to treatment changes in a substantial number of patients (1, 7, 8). Since this heterogeneity increases over time and under treatment pressure (7), repetitive analyses of the characteristics of metastatic tumor cells are likely to offer better guidance of treatment choices than characterization of the primary tumor. Unfortunately, metastatic tissue is often hard to obtain and only possible through invasive procedures.

Circulating tumor cells (CTCs) are tumor cells found in the peripheral blood and are thought to better represent the actual or clinically relevant metastatic tissue burden than the primary tumor does, in particular in those patients whose primary tumors have been removed several years prior to diagnosis of MBC. The CTC count has shown to be a powerful prognostic factor in MBC and a rise or decline in CTC count after the first cycle of systemic therapy is an early predictor of outcome (9-12). Additionally, CTC characterization holds great promise and for that purpose, several techniques to molecularly characterize CTCs for drug target expression (13-15), mutations (16) and gene expression (17-19) have been developed. CTCs however occur in relatively low numbers in patients with MBC and, even after the epithelial cell adhesion molecule (EpCAM)-based enrichment of the CellSearch® system, they need to be identified and characterized amongst approximately a thousand of remaining leukocytes (20). This greatly hinders the interpretation of results from techniques non-selective for tumor cells such as quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) on whole lysates. Nevertheless, by focusing on genes that are not, or only at a much lower level, expressed by leukocytes, we have previously shown that the expression levels of 96 genes in CTCs can be quantified in MBC patients through qRT-PCR (18).

In this study, we aim to quantify this panel of 96 genes in CTCs of MBC patients with ER-expressing primary tumors prior to start of first-line therapy with an aromatase inhibitor (AI) in order to identify a CTC predictor discriminating between good and poor responders.

METHODS

ETHICS STATEMENT

This study has been approved by the medical ethics committee of the Erasmus MC Rotterdam, The Netherlands and local Institutional Review Boards (ethics boards of Oncology Center GZA Hospitals Sint-Augustinus, Antwerp, Belgium; Ikazia Hospital, Rotterdam, The Netherlands; Sint Franciscus Gasthuis, Rotterdam) (METC 2006–248 and METC 2009–405). All patients gave their written informed consent.
We adhered to the Reporting Recommendations for Tumor Marker Prognostic Studies wherever possible (21).

COLLECTION OF BLOOD SAMPLES AND CHARACTERISTICS OF RECRUITED PATIENT COHORT

MBC patients had been included between October 2008 and August 2012 in 5 hospitals. From 78 MBC patients who were not previously treated for MBC and prior to start of first-line AI therapy (irrespective of type), 2 x 7.5 mL blood samples were prospectively drawn for CTC enumeration and isolation. Due to insufficient RNA quality and/or quantity and/or lack of expression of previously described CTC-specific genes (18) (for details see next), 33 (42%) samples were excluded, providing 45 patients for further analysis (Additional file 1: Figure S1). Detailed clinicopathological information for these 45 patients is provided in Table 1.

In order to be able to decipher whether obtained results from this AI-treated patient cohort are of prognostic or predictive nature, we used an independent patient cohort composed of 71 MBC patients that received other types of first-line therapy. Of these, 21 patients were treated with chemotherapy, 40 patients with chemotherapy combined with targeted therapy, and 10 patients with tamoxifen therapy. This patient cohort had been extracted from MBC patients described in our recently published study in which the same techniques for CTC enrichments and gene expression determination were applied (22).

TABLE 1. Patients and their clinico-pathological characteristics

Characteristic	No. of patients	%
All patients	**45**	**100%**
Time between primary surgery and CTC sampling (DFI)		
≤ 5 years	16	36%
>5 years	21	47%
Primary not removed	8	18%
Age at CTC sampling		
≤ 50 years	4	9%
>50 years	41	91%
Menopausal status		
Premenopausal	2	4%
Postmenopausal	43	96%
Histologic grade (Bloom-Richardson)		
I, well differentiated	5	11%
II, moderately differentiated	23	51%
III, poorly differentiated	4	9%
Unknown	13	29%
Pathological tumor size		
pT1, ≤2 cm	20	44%
pT2-4, >2 cm	22	49%
Unknown	3	7%
Lymph nodes involved		
No	14	31%
Yes	27	60%
Unknown	4	9%
ERα status[†]		
Negative	1	2%
Positive	44	98%
PgR status[†]		
Negative	5	11%
Positive	36	80%
Unknown	4	9%

TABLE 1. (continued)

Characteristic	No. of patients	%
HER2/neu status†		
Negative	37	82%
Positive	3	7%
Unknown	5	11%
Histological type		
Lobular	13	29%
Ductal	28	62%
Ductolobular	3	7%
Ductal, signet-cell	1	2%
Adjuvant chemotherapy		
No	31	69%
Yes	14	31%
Adjuvant hormonal therapy		
No	24	53%
Yes	21	47%
Any adjuvant therapy		
No	22	49%
Yes	23	51%
Site of metastasis		
Visceral	5	11%
Non-visceral	26	58%
Both	14	31%
1st line treatment		
Anastrozol	15	33%
Letrozol	16	36%
Exemestane	14	31%
Median progression-free survival (PFS in days; range)*	358 (14 - 1255)	
Median baseline CTC count (range in 7.5 mL blood)	8 (0 - 32,492)	

† As retrieved from pathology reports

* Also includes censoring data from patients censored at last follow-up date

ENUMERATION OF CTCS

In order to isolate CTCs for CTC enumeration, 7.5 mL blood was drawn in CellSave tubes (Veridex™ LCC, Raritan, NJ, USA) and processed on the CellTracks AutoPrep System by using the CellSearch Epithelial Cell Kit (both Veridex LCC). CTC enumeration was performed on the CellTracks Analyzer (Veridex LCC) according to the manufacturer's instructions and as described previously (23-25).

MRNA ISOLATION FROM CTCS, QRT-PCR AND QUANTIFICATION OF GENE TRANSCRIPTS

Together with the blood samples for CTC enumeration, another 7.5 mL blood of the same patients was drawn in EDTA tubes. These samples were enriched for CTCs on the CellTracks AutoPrep System using the CellSearch Profile Kit (Veridex LCC). Isolated cells were lysed by adding 250 µL of Qiagen AllPrep DNA/RNA Micro Kit Lysis Buffer (RLT+ lysis buffer) (Qiagen BV, Venlo, The Netherlands) and immediately stored at -80 °C until RNA isolation was performed with the AllPrep DNA/RNA Micro Kit (Qiagen) according to the manufacturer's instructions and as previously described (18).

The generation of cDNA from isolated RNA from CTCs and subsequent pre-amplification and TaqMan-based PCR analysis were performed as described before (20). The 96 measured mRNA transcripts have previously been selected and validated based on their clinical relevance and potential CTC-specificity (18, 20).

REFERENCE GENES, DATA NORMALIZATION, AND QUALITY CONTROL

The procedure of data normalization and quality control was performed as previously described (18, 20). In short, relative expression levels were quantified by using the delta Ct method, which is the difference between the average Ct of the reference genes *HMBS*, *HPRT1*, and *GUSB* and the Ct of the target genes. Samples that were able to generate a signal within the chosen cut-off set at 26 Ct of the average of the reference genes were considered of sufficient quality and quantity to be included in the study and quantified for the levels of the remaining 93 target genes. By the use of this threshold, 5 of our initial 78 CTC samples (6%) were excluded from further analysis.

Finally, samples were checked for sufficient expression levels of a 12-gene mRNA cluster that has previously been determined as epithelial-specific and associated with the presence of CTCs (18). Due to lack of sufficient expression of these genes and our aim to generate a CTC-specific predictor, another 28 CTC samples (36%) were excluded from further analysis.

STATISTICAL ANALYSIS

Statistical analyses were done with the STATA statistical package, release 12.0 (STATA Corp., College Station, TX). Primary endpoint was progression-free survival (PFS), defined as the time elapsed between start of first-line treatment with AI and clinical and/or radiological progression or death, whichever came first. Patients who were alive and had not progressed were censored at the last follow-up date, which was at least 9 months after start of 1st line therapy. Those patients with progression or death <9 months were considered as poor responders. This 9-month cut-off was chosen based on the median

PFS for first-line therapy in MBC patients as reported in the literature (26, 27). In all 45 eligible patients, a leave-one-out-cross validation (LOOCV) was conducted using the Support Vector Machines (SVM) method within Biometric Research Branc ArrayTools (http://linus.nci.nih.gov/BRB-ArrayTools.html) after selecting the top 75% most variable genes from the 93 genes described above. With this LOOCV method, a gene signature was generated that consisted out of the most differentially expressed genes that were identified in the individual predictions and best predicted the left-out sample. A panel of 8 genes was identified that performed best in predicting the poor responding patients. The SVM method proved superior compared to the other prediction algorithms; based on 100 permutations, SVM was the only method with a significant P-value of 0.01. Cluster 3.0 and TreeView (http://bonsai.hgc.jp/~mdehoon/software/cluster/clustersetup.exe and http://jtreeview.sourceforge.net/ [28]) were used to cluster the samples according to the gene expression values of these 8 genes and to visualize the results. Survival curves were generated using the Kaplan-Meier method and a logrank test was used to test for differences. All statistical tests were 2-sided with $P < 0.05$ considered statistically significant.

RESULTS

PATIENT CHARACTERISTICS

Characteristics of the 45 patients who were eligible for our CTC-specific analyses to explore differentially expressed genes between good and poor responders are listed in Table 1. One patient was described to have an ER-negative primary tumor but received hormonal treatment in both adjuvant and first-line setting due to PR-positivity. Median baseline CTC count in the 45 patient cohort was 8 (range 0 – 32,492 CTCs/7.5 mL blood). The extremely high CTC count of 32,492 was assessed in a patient who did not respond to treatment and died within one month after treatment initiation due to progression of disease. The 9-month cutoff as based on literature data on the median PFS in first-line MBC patients (26, 27) was well-chosen considering the median PFS of 11.8 months (range 0 – 41.3 months) in our 45 patient cohort.

8-GENE CTC PROFILE PREDICTS FOR OUTCOME TO TREATMENT

Of the 45 patients, 19 patients were classified as poor responders due to progression of disease or death <9 months whereas the remaining 26 patients were considered good responders. A LOOCV was performed in this cohort yielding an 8-gene predictor in which each gene had a univariate *P*-value of <0.1 (Table 2). Application of this 8-gene CTC profile resulted in 16 patients with an unfavorable profile and were thus predicted to be poor responders. Twelve of them truly showed resistance to therapy <9 months (disease progression or death) and four did not, resulting in a sensitivity of 63% and a positive predictive value (PPV) of 75% (Table 3). Applying the profile, 29 patients had a favorable profile and were thus predicted not to show progressive disease <9 months. Of these, 22 indeed did not fail treatment <9 months rendering a specificity of 85% and a negative predictive value (NPV) of 76%.

TABLE 2. Significantly differentially expressed genes between 45 good and poor responders

Gene	*P*-value	t-value
TWIST1	0,001	-2,879
KRT81	0,018	-2,453
PTRF	0,029	-2,024
EEF1A2	0,031	-1,895
PTPRK	0,049	-1,793
EGFR	0,065	-1,701
CXCL14	0,080	2,229
ERBB3	0,096	2,260

A negative t-value corresponds to higher expression in poor responding patients; a positive t-value to higher expression in good responding patients.

TABLE 3. Test performance

	8-gene CTC profile		
PFS <9 months	Favorable	Unfavorable	**Total**
No	22	4	26
Yes	7	12	19
Total	29	16	45

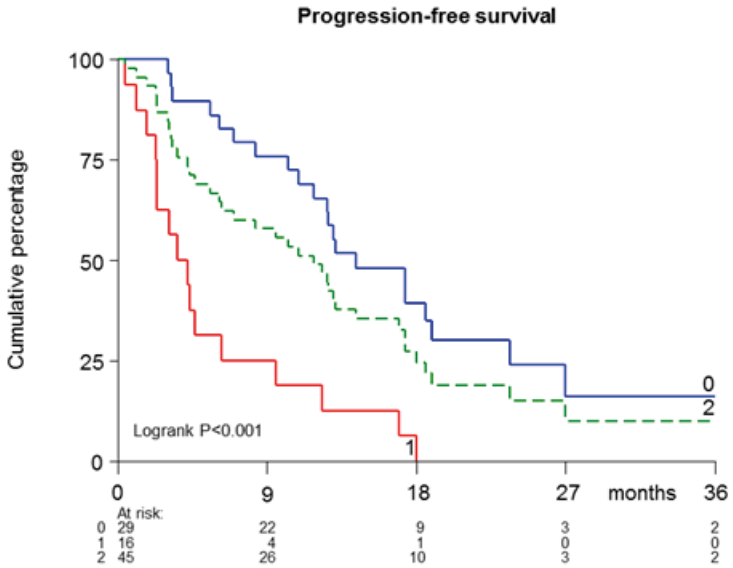
Pearson's X^2 statistic 10,93

P <0.001

The Kaplan-Meier curves for PFS of the predicted good and poor responding patients according to the 8-gene CTC predictor are shown in Figure 1 and were statistically different (Logrank $P < 0.001$).

In univariate analysis, the 8-gene CTC predictor was significantly associated with PFS (HR 4.40 [95% CI: 2.17-8.92], $P < 0.001$). When including the traditional predictive factors, disease-free interval (DFI), which was defined as the time between primary surgery and CTC sampling, the dominant site of relapse, and the CTC count at baseline in a multivariate analysis, only the 8-gene CTC-profile was an independent predictor of PFS (HR 4.59 [95% CI: 2.16-9.75], $P < 0.001$) (Table 4). The CTC count at baseline was not associated with PFS in this 45 patient cohort, but showed to be significant in the total cohort of 78 patients (HR 2.47 [95% CI: 1.43-4.27], $P = 0.001$) (Additional file 2: Figure S2).

FIGURE 1. Kaplan-Meier curve for patients as defined by the 8-gene CTC predictor. Blue (0): favorable profile; red (1): unfavorable profile; green (2): total cohort (*N*=45)



HIERARCHICAL CLUSTERING TO IDENTIFY CLUSTERS OF PATIENTS ACCORDING TO THE 8-GENE CTC PREDICTOR

Two-dimensional average linkage hierarchical cluster analysis (28) was performed to compare the difference in gene expression of the 8 identified genes in our 45 patients. This analysis resulted in a clustering of 2 major and 5 minor groups of patients in which cluster 1 mainly contained the good responders (10 out of 12), whereas cluster 2 consisted of both good and poor responders (Figure 2). In this cluster, however, a subcluster existed that, with 10 out of 12, predominantly contained poor responders with higher expression of most of the identified 8 genes.

TESTING THE 8-GENE CTC PROFILE IN AN INDEPENDENT DIFFERENTLY TREATED PATIENT COHORT

Having identified the 8-gene CTC profile in AI-treated patients, it was assessed whether this signature was prognostic or predictive by investigating the association between this profile and outcome in an independent patient cohort composed of 71 MBC patients that received other first-line therapies than AI. Of these, 21 patients were treated with chemotherapy, 40 with chemotherapy combined with a type of targeted therapy such as trastuzumab, and 10 with tamoxifen therapy. Of this group, 35 patients had a PFS of less than 9 months and were therefore classified as having a poor outcome. Application of the 8-gene CTC profile resulted in 33 patients with a favorable CTC profile. The CTC profile however, could not properly discriminate the patients with a good versus those with a poor outcome (P = 0.899; Table 5).

TABLE 4. Predictive value of the 8-gene CTC profile in uni- and multivariate analysis

Factor of base model	Univariate analysis			Multivariate analysis		
	PFS			PFS		
	HR	**95% CI**	***P***	**HR**	**95% CI**	***P***
<5 vs. ≥5 CTCs at baseline	1.11	0.57-2.15	0.753	1.31	0.65-2.62	0.455
Disease-free interval*	1.09	0.74-1.61	0.653	0.95	0.62-1.43	0.790
Dominant site of relapse**	1.34	0.69-2.58	0.384	1.11	0.56-2.18	0.768
8-gene CTC profile	4.40	2.17-8.92	<0.001	4.59	2.16-9.75	<0.001

* Defined as the time between primary surgery and CTC sampling and analyzed in 3 groups: ≤5 years (*N*= 12), >5 years (*N*= 21) and metastatic disease upon diagnosis (*N*= 12)
** Divided into non-visceral vs. visceral metastases

FIGURE 2. Unsupervised hierarchical cluster analysis comparing the 8-gene CTC predictor in 45 MBC patients treated with first-line AI therapy. Each horizontal row represents a gene, and each vertical column corresponds to a sample. Red color indicates a mRNA expression level above the median level, black color indicates a median expression level, and green color indicates an expression level below the median level of the assay as measured in all 45 samples. The number of CTCs as established by the CellSearch Epithelial Kit is depicted below the figure. Blue: good responder; red: poor responder. CTC count: blue: <5 CTCs; red: ≥5 CTCs

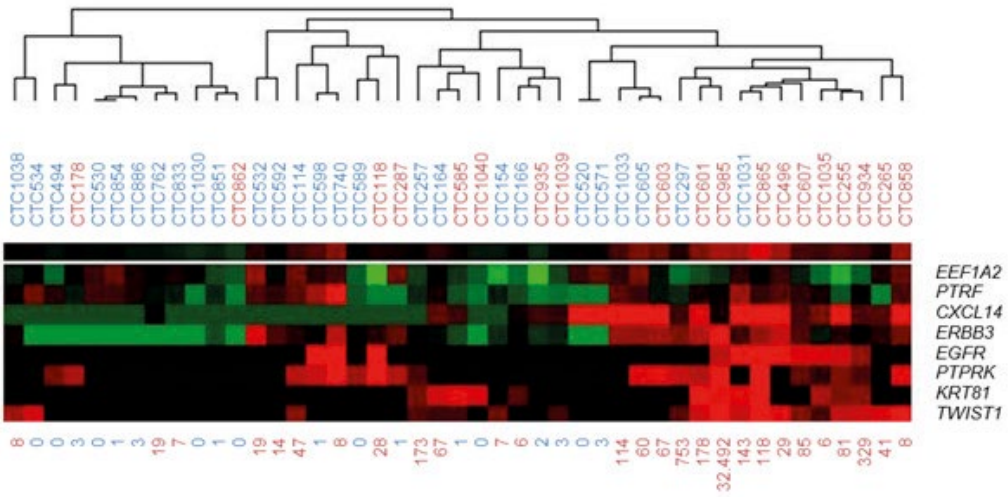


TABLE 5. Test performance of the 8-gene CTC predictor in 71 patients not treated with AI therapy

	8-gene CTC profile		
PFS <9 months	Favorable	Unfavorable	**Total**
No	16	19	35
Yes	17	19	36
Total	33	38	71
Pearson's X^2 statistic	0.016		
P	0.899		

DISCUSSION

Characterization of CTCs holds great promise to predict response to treatment and to gain more insight into mechanisms underlying resistance to systemic anti-tumor agents. Although whole transcriptome analysis would be most preferable, isolation of CTCs by the CellSearch technique does not result in pure fractions of CTCs but only in fractions enriched for CTCs in which an overload of leukocytes is still present. This makes interpretation of whole transcriptome analysis impossible since only techniques yielding pure CTC fractions would allow such analyses. We have previously shown to be able to measure mRNA expression levels of multiple epithelial genes in CTCs enriched by CellSearch (18). By using these selected genes and applying the same technique, the current study demonstrates the ability of using CTC characterization as a predictor for response to endocrine therapy. To our best knowledge, this is the first study that has generated an unique CTC-based gene expression panel that is able to distinguish good and poor responders to first-line AI therapy. From a clinical point of view, it is probably more relevant to identify the poor rather than the good responding patients, since these patients might benefit more from another treatment. Our identified 8-gene CTC profile however performed better in predicting the good responders, since the specificity of the predictor outperforms its sensitivity (85% vs. 63%; Table 3). Nevertheless, this could still impact clinical decision making since good responding patients could undergo less intensive follow-up strategies and fewer laboratory procedures which is not only less demanding for patients but can also reduce health care costs.

In order to explore whether this signature associated with outcome in AI-treated patients is prognostic or predictive, we tested the profile in CTCs of a group of 71 patients who were treated with types of systemic treatments other than AI including chemotherapy (*N*= 21), chemotherapy combined with a type of targeted therapy (*N*= 40), or tamoxifen therapy (*N*= 10). In contrast to the AI-treated patients, the 8-gene CTC profile could not discriminate patients with a good versus those with a poor outcome in this group of patients (*P* = 0.899; Table 5). Although this is not a true validation of the test, it strongly supports that the identified profile is predictive for outcome to AI therapy and not for outcome to other agents. It needs to be underscored that the identified CTC profile has been obtained in a small number of patients for which an LOOCV procedure to reveal such a profile is commonly

applied. It important to realize that such an approach bears the risk of overfitting the data as a consequence of which validation in an independent patient cohort is needed before implementation in clinical practice.

The development of a CTC-specific predictor required exclusion of patients who lacked sufficient expression of epithelial-specific genes. These are mainly patients with no or few counted CTCs and are therefore more likely to have a longer PFS which might have biased our patient set (9). Although most characteristics do not show differences between in- and excluded patients (Additional file 3: Table S1), the median PFS in the 33 excluded patients was 548 (40-1694) days which significantly differs from the median PFS of 358 (14-1255) days in the 45 included patients (Logrank $P < 0.001$). This exclusion criterion highly affected the number of patients available for further analysis. The low number of remaining patients might be the reason for the insignificant association between the CTC count at baseline (divided in <5 vs. ≥5 CTCs) and PFS. In the total cohort of 78 patients, CTC count was significantly related to PFS (Additional file 2: Figure S2). Since cohorts with few patients cannot be divided into independent discovery and validation sets, resampling the original data through cross-validation is statistically the best method (29).

Amongst the 8 genes that we found to be associated with outcome to AI therapy through LOOCV, was the epithelial marker *KRT81*. Many cytokeratins are highly expressed in both normal and tumor epithelium in which the pattern of expression can be used to identify the tissue of origin (30). Not much is known about this specific cytokeratin and why high expression would lead to a worse outcome. Mutations in *KRT81* have been described in monilethrix, a condition in which patients develop diffuse hypothrichosis (31).

CXCL14 and *ERBB3* were the only genes that were more abundantly expressed in the good responding patients. This is discordant to what is currently known in primary tumor tissue with respect to both genes. The published literature, however, only considers gene expression in primary tumors which cannot easily be extrapolated to CTCs. *CXCL14* is a chemokine that has been shown to be upregulated in tumor myoepithelial cells and enhances the proliferation, migration, and invasion of epithelial cells after binding to their receptors (32). Expression of *ERBB3* has, similar to *EGFR* in our CTC predictor, previously been associated with endocrine therapy resistance when highly expressed in primary tumor tissue (33, 34). The predictor also contained high expression of *PTRF* and *EEF1A2* to be associated with poor outcome. This is in contrast with previously published literature in which *PTRF* has been shown to interact with *pS2/TTF1* (35) which on its turn needs ER as key transcriptional factor in order to be expressed (36) and is associated with a better clinical outcome in breast cancer (37-39). *EEF1A2* is an eukaryotic elongation factor of which its expression downregulates through interaction with protein p16(INK4a) leading to inhibition of cancer cell growth (40). It is mainly known as a potential oncogene in ovarian cancer in which its expression enhances cell growth in vitro (41). Overexpression of *EEF1A2* has also been seen in breast tumors (42) and it is one of the genes in the 76-gene signature as identified in the ER-positive subset

of 115 primary breast tumors that represent a strong prognostic factor for patients at high risk for developing metastases (43, 44). With respect to the other genes of the predictor, *PTPRK* belongs to the group of protein-tyrosine phosphatases (PTPs) that control tyrosine phosphorylation. PTPs regulate the signaling of growth-factor receptors and can, when deregulated, be associated with tumorigenesis (45). Deregulation of PTPs can result in both their up- and downregulation, which can explain the discordance between our established association between high expression of *PTPRK* and poor outcome to AI therapy, while decreased expression of *PTPRK* has previously been related to poor prognosis in MBC suggesting a more tumor suppressive role (46). *TWIST1*, at last, is a transcription factor that is one of the most widely known factors to be involved in the process of epithelial-to-mesenchymal-transition (EMT). Its overexpression has been associated with endocrine therapy resistance due to downregulation of ER promoter activity (47). Moreover, through direct repression of E-cadherin cells and activation of mesenchymal markers, *TWIST1* plays an essential role in tumor metastasis (48). The appearance of *TWIST1* in our 8-gene CTC predictor is remarkable since our applied CTC isolation method relies on an EpCAM-based enrichment step and tumor cells undergoing EMT might become EpCAM-negative (49). The dependency on EpCAM-expression by CTCs renders the CellSearch method therefore not the best method to capture all CTCs, but it is still the only FDA-cleared method which will enable its implementation and obtained results in clinical studies. In addition, whether EpCAM loss always accompanies EMT is still under debate (50).

Although ER is amongst the 93 target genes that were measured, its mRNA expression in this study was not associated with outcome to AI therapy. Several techniques have been explored to determine ER expression in CTC, but so far, none of these studies could show an association with outcome (reviewed in (19)). Recently, Babayan et al. have demonstrated the possibility of measuring ER protein expression in single CTCs through immunofluorescence. This study revealed that CTCs of individual MBC patients with ER-positive primary tumors are frequently a heterogeneous population consisting of both ER-positive and ER-negative CTCs (51). Similar to primary tumor tissue, the percentage of ER-positive CTCs may be the best parameter associated with outcome rather than ER mRNA expression of the total CTC fraction as was measured in our study.

CONCLUSION

In conclusion, we have here defined an 8-gene expression predictor established in CTCs that is associated with outcome to first-line AI therapy in MBC patients. Importantly, before the results of the current study can be implemented, an independent patient cohort is warranted to validate the results found here. Nevertheless, this study underscores the enormous potential that molecular characterization of CTCs has.

REFERENCES

1. Thompson AM, Jordan LB, Quinlan P, Anderson E, Skene A, Dewar JA, et al. Prospective comparison of switches in biomarker status between primary and recurrent breast cancer: the Breast Recurrence In Tissues Study (BRITS). Breast Cancer Res. 2010;12(6):R92.
2. Gong Y, Han EY, Guo M, Pusztai L, Sneige N. Stability of estrogen receptor status in breast carcinoma: a comparison between primary and metastatic tumors with regard to disease course and intervening systemic therapy. Cancer. 2011;117(4):705-13.
3. Amir E, Miller N, Geddie W, Freedman O, Kassam F, Simmons C, et al. Prospective study evaluating the impact of tissue confirmation of metastatic disease in patients with breast cancer. J Clin Oncol. 2012;30(6):587-92.
4. Pancholi S, Lykkesfeldt AE, Hilmi C, Banerjee S, Leary A, Drury S, et al. ERBB2 influences the subcellular localization of the estrogen receptor in tamoxifen-resistant MCF-7 cells leading to the activation of AKT and RPS6KA2. Endocr Relat Cancer. 2008;15(4):985-1002.
5. Miller TW, Balko JM, Arteaga CL. Phosphatidylinositol 3-kinase and antiestrogen resistance in breast cancer. J Clin Oncol. 2011;29(33):4452-61.
6. Reijm EA, Jansen MP, Ruigrok-Ritstier K, van Staveren IL, Look MP, van Gelder ME, et al. Decreased expression of EZH2 is associated with upregulation of ER and favorable outcome to tamoxifen in advanced breast cancer. Breast Cancer Res Treat. 2011;125(2):387-94.
7. Campbell PJ, Yachida S, Mudie LJ, Stephens PJ, Pleasance ED, Stebbings LA, et al. The patterns and dynamics of genomic instability in metastatic pancreatic cancer. Nature. 2010;467(7319):1109-13.
8. Xiao C, Gong Y, Han EY, Gonzalez-Angulo AM, Sneige N. Stability of HER2-positive status in breast carcinoma: a comparison between primary and paired metastatic tumors with regard to the possible impact of intervening trastuzumab treatment. Ann Oncol. 2011;22(7):1547-53.
9. Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med. 2004;351(8):781-91.
10. Cristofanilli M, Hayes DF, Budd GT, Ellis MJ, Stopeck A, Reuben JM, et al. Circulating tumor cells: a novel prognostic factor for newly diagnosed metastatic breast cancer. J Clin Oncol. 2005;23(7):1420-30.
11. Hayes DF, Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Miller MC, et al. Circulating tumor cells at each follow-up time point during therapy of metastatic breast cancer patients predict progression-free and overall survival. Clin Cancer Res. 2006;12(14 Pt 1):4218-24.
12. Bidard FC, Peeters DJ, Fehm T, Nole F, Gisbert-Criado R, Mavroudis D, et al. Clinical validity of circulating tumour cells in patients with metastatic breast cancer: a pooled analysis of individual patient data. Lancet Oncol. 2014;15(4):406-14.
13. Attard G, Swennenhuis JF, Olmos D, Reid AH, Vickers E, A'Hern R, et al. Characterization of ERG, AR and PTEN gene status in circulating tumor cells from patients with castration-resistant prostate cancer. Cancer Res. 2009;69(7):2912-8.
14. Fehm T, Muller V, Aktas B, Janni W, Schneeweiss A, Stickeler E, et al. HER2 status

of circulating tumor cells in patients with metastatic breast cancer: a prospective, multicenter trial. Breast Cancer Res Treat. 2010;124(2):403-12.
15. Riethdorf S, Muller V, Zhang L, Rau T, Loibl S, Komor M, et al. Detection and HER2 expression of circulating tumor cells: prospective monitoring in breast cancer patients treated in the neoadjuvant GeparQuattro trial. Clin Cancer Res. 2010;16(9):2634-45.
16. Gasch C, Bauernhofer T, Pichler M, Langer-Freitag S, Reeh M, Seifert AM, et al. Heterogeneity of epidermal growth factor receptor status and mutations of KRAS/PIK3CA in circulating tumor cells of patients with colorectal cancer. Clin Chem. 2013;59(1):252-60.
17. Smirnov DA, Zweitzig DR, Foulk BW, Miller MC, Doyle GV, Pienta KJ, et al. Global gene expression profiling of circulating tumor cells. Cancer Res. 2005;65(12):4993-7.
18. Sieuwerts AM, Mostert B, Bolt-de Vries J, Peeters D, de Jongh FE, Stouthard JM, et al. mRNA and microRNA expression profiles in circulating tumor cells and primary tumors of metastatic breast cancer patients. Clin Cancer Res. 2011;17(11):3600-18.
19. Onstenk W, Gratama JW, Foekens JA, Sleijfer S. Towards a personalized breast cancer treatment approach guided by circulating tumor cell (CTC) characteristics. Cancer Treat Rev. 2013.
20. Sieuwerts AM, Kraan J, Bolt-de Vries J, van der Spoel P, Mostert B, Martens JW, et al. Molecular characterization of circulating tumor cells in large quantities of contaminating leukocytes by a multiplex real-time PCR. Breast Cancer Res Treat. 2009;118(3):455-68.
21. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM, et al. Reporting recommendations for tumor marker prognostic studies (REMARK). J Natl Cancer Inst. 2005;97(16):1180-4.
22. Mostert B, Sieuwerts AM, Kraan J, Bolt-de Vries J, van der Spoel P, van Galen A, et al. Gene expression profiles in circulating tumor cells to predict prognosis in metastatic breast cancer patients. Ann Oncol. 2015;26(3):510-6.
23. Sieuwerts AM, Kraan J, Bolt J, van der Spoel P, Elstrodt F, Schutte M, et al. Anti-epithelial cell adhesion molecule antibodies and the detection of circulating normal-like breast tumor cells. J Natl Cancer Inst. 2009;101(1):61-6.
24. Mostert B, Kraan J, Bolt-de Vries J, van der Spoel P, Sieuwerts AM, Schutte M, et al. Detection of circulating tumor cells in breast cancer may improve through enrichment with anti-CD146. Breast Cancer Res Treat. 2011;127(1):33-41.
25. Kraan J, Sleijfer S, Strijbos MH, Ignatiadis M, Peeters D, Pierga JY, et al. External quality assurance of circulating tumor cell enumeration using the CellSearch((R)) system: a feasibility study. Cytometry B Clin Cytom. 2011;80(2):112-8.
26. Gennari A, Conte P, Rosso R, Orlandini C, Bruzzi P. Survival of metastatic breast carcinoma patients over a 20-year period: a retrospective analysis based on individual patient data from six consecutive studies. Cancer. 2005;104(8):1742-50.
27. Kiely BE, Soon YY, Tattersall MH, Stockler MR. How long have I got? Estimating typical, best-case, and worst-case scenarios for patients starting first-line chemotherapy for metastatic breast cancer: a systematic review of recent randomized trials. J Clin Oncol. 2011;29(4):456-63.
28. Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-

wide expression patterns. Proc Natl Acad Sci U S A. 1998;95(25):14863-8.
29. Molinaro AM, Simon R, Pfeiffer RM. Prediction error estimation: a comparison of resampling methods. Bioinformatics. 2005;21(15):3301-7.
30. Moll R, Franke WW, Schiller DL, Geiger B, Krepler R. The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. Cell. 1982;31(1):11-24.
31. Ferrando J, Galve J, Torres-Puente M, Santillan S, Nogues S, Grimalt R. Monilethrix: A New Family with the Novel Mutation in KRT81 Gene. Int J Trichology. 2012;4(1):53-5.
32. Allinen M, Beroukhim R, Cai L, Brennan C, Lahti-Domenici J, Huang H, et al. Molecular characterization of the tumor microenvironment in breast cancer. Cancer Cell. 2004;6(1):17-32.
33. Nicholson RI, Hutcheson IR, Harper ME, Knowlden JM, Barrow D, McClelland RA, et al. Modulation of epidermal growth factor receptor in endocrine-resistant, oestrogen receptor-positive breast cancer. Endocr Relat Cancer. 2001;8(3):175-82.
34. Morrison MM, Hutchinson K, Williams MM, Stanford JC, Balko JM, Young C, et al. ErbB3 downregulation enhances luminal breast tumor response to antiestrogens. J Clin Invest. 2013;123(10):4329-43.
35. Jansa P, Mason SW, Hoffmann-Rohrer U, Grummt I. Cloning and functional characterization of PTRF, a novel protein which induces dissociation of paused ternary transcription complexes. EMBO J. 1998;17(10):2855-64.
36. Metivier R, Penot G, Hubner MR, Reid G, Brand H, Kos M, et al. Estrogen receptor-alpha directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. Cell. 2003;115(6):751-63.
37. Foekens JA, Rio MC, Seguin P, van Putten WL, Fauque J, Nap M, et al. Prediction of relapse and survival in breast cancer patients by pS2 protein status. Cancer Res. 1990;50(13):3832-7.
38. Foekens JA, van Putten WL, Portengen H, de Koning HY, Thirion B, Alexieva-Figusch J, et al. Prognostic value of PS2 and cathepsin D in 710 human primary breast tumors: multivariate analysis. J Clin Oncol. 1993;11(5):899-908.
39. Foekens JA, Portengen H, Look MP, van Putten WL, Thirion B, Bontenbal M, et al. Relationship of PS2 with response to tamoxifen therapy in patients with recurrent breast cancer. Br J Cancer. 1994;70(6):1217-23.
40. Lee MH, Choi BY, Cho YY, Lee SY, Huang Z, Kundu JK, et al. Tumor suppressor p16(INK4a) inhibits cancer cell growth by downregulating eEF1A2 through a direct interaction. J Cell Sci. 2013;126(Pt 8):1744-52.
41. Pinke DE, Kalloger SE, Francetic T, Huntsman DG, Lee JM. The prognostic significance of elongation factor eEF1A2 in ovarian cancer. Gynecol Oncol. 2008;108(3):561-8.
42. Tomlinson VA, Newbery HJ, Wray NR, Jackson J, Larionov A, Miller WR, et al. Translation elongation factor eEF1A2 is a potential oncoprotein that is overexpressed in two-thirds of breast tumours. BMC Cancer. 2005;5:113.
43. Wang Y, Klijn JG, Zhang Y, Sieuwerts AM, Look MP, Yang F, et al. Gene-expression profiles to predict distant metastasis of lymph-node-negative primary breast cancer. Lancet. 2005;365(9460):671-9.
44. Zhang Y, Sieuwerts AM, McGreevy M, Casey G, Cufer T, Paradiso A, et al. The 76-gene

signature defines high-risk patients that benefit from adjuvant tamoxifen therapy. Breast Cancer Res Treat. 2009;116(2):303-9.
45. Alonso A, Sasin J, Bottini N, Friedberg I, Friedberg I, Osterman A, et al. Protein tyrosine phosphatases in the human genome. Cell. 2004;117(6):699-711.
46. Sun PH, Ye L, Mason MD, Jiang WG. Protein tyrosine phosphatase kappa (PTPRK) is a negative regulator of adhesion and invasion of breast cancer cells, and associates with poor prognosis of breast cancer. J Cancer Res Clin Oncol. 2013;139(7):1129-39.
47. Vesuna F, Lisok A, Kimble B, Domek J, Kato Y, van der Groep P, et al. Twist contributes to hormone resistance in breast cancer by downregulating estrogen receptor-alpha. Oncogene. 2012;31(27):3223-34.
48. Yang J, Mani SA, Donaher JL, Ramaswamy S, Itzykson RA, Come C, et al. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. Cell. 2004;117(7):927-39.
49. Iwatsuki M, Mimori K, Yokobori T, Ishi H, Beppu T, Nakamori S, et al. Epithelial-mesenchymal transition in cancer development and its clinical significance. Cancer Sci. 2010;101(2):293-9.
50. van der Gun BT, Melchers LJ, Ruiters MH, de Leij LF, McLaughlin PM, Rots MG. EpCAM in carcinogenesis: the good, the bad or the ugly. Carcinogenesis. 2010;31(11):1913-21.
51. Babayan A, Hannemann J, Spotter J, Muller V, Pantel K, Joosse SA. Heterogeneity of estrogen receptor expression in circulating tumor cells from metastatic breast cancer patients. PLoS One. 2013;8(9):e75038.

SUPPLEMENTARY MATERIAL

https://drive.google.com/open?id=0B_eW3FoE0HzYdEJNZHlUcWx6ODg

CHAPTER 7

Discussion of the thesis

DISCUSSION OF THE THESIS

The field of oncology is constantly moving and we continuously obtain more insight into carcinogenesis, the development of metastases, and therapy resistance. Meanwhile, measures for prevention and tailored treatment are evolving. However, despite all the progress achieved, therapy resistance remains a constant hurdle in the treatment of cancer patients. The complexity of the mechanisms involved in the development of metastases and drug response hinders the discovery of good predictive and prognostic factors.

Overall, the implementation of new cancer therapies has improved the survival and quality of life of cancer patients but the incidence and prevalence is dramatically increasing resulting in high health care costs. Since we know that only a subset of patients will benefit from a certain treatment it is crucial to select the right patient for the right treatment. Better selection of patients and treatment is not only necessary to improve cancer care affordability but will also lead to better quality of life since the trade-off between efficacy and toxicity will become more favorable. Improved prognostic and predictive factors will not only estimate life expectancy more adequately but will thus also result in true personalized medicine.

The current prognostic and predictive factors for breast cancer encompasses tumor characteristics as size, lymph node status and molecular profiles. The discovery of the role of ER, PR and HER2 in the tumor biology of breast cancer and the development of therapies targeting these factors have dramatically changed clinical decision making in breast cancer treatment and favorably changed the prognosis.

ER targeting therapies form the mainstay of treatment for patients with ER-positive breast cancer. Although the majority of breast tumors express the ER, approximately 30% of the patients with ER-positive advanced disease never respond to endocrine therapy and all initial responders will eventually develop resistance (1). Therefore, there is a high need for markers to identify patients likely to benefit from therapy for which a better insight into the mechanisms conferring endocrine treatment resistance is required.

In this thesis, we have investigated several factors involved in endocrine therapy resistance measured in both cell line models as well as primary tumors and circulating tumor cells (CTC) from patients.

EZH2 INHIBITION AND THERAPY

Over the years, multiple factors accounting for resistance to endocrine therapy have been revealed. The development of new drugs and new combinations is usually based on these factors. We have described Enhancer of Zeste Homolog 2 (EZH2) as a potential new marker to better predict response to tamoxifen. A genome-wide profiling study in metastatic breast cancer (MBC) patients treated with first-line tamoxifen therapy revealed a higher EZH2 expression in patients that had early progressive disease (2).

EZH2 is the catalytic subunit of the Polycomb Repressor Complex 2 (PRC2) which also contains EED, SUZ12 and RBBP4/RbAp48/NURF55 that are required for the histone methyltransferase activity that EZH2 exerts. EZH2 is a member of the Polycomb group (PcG) proteins and mediates di- and trimethylation of lysine residue 27 on histone 3 (H3K27). This modification results in chromatin compaction and transcriptional silencing (3). As a consequence, EZH2 plays an important role in embryonic development, cell differentiation, but also carcinogenesis (4-6). Overexpression of EZH2 was first found in prostate cancer and linked to a poorer prognosis (7). Nowadays, it is known that overexpression of EZH2 also appears in multiple tumor types like lymphomas (8, 9), urogenital tract tumors (10-12), ovarian cancer (13) and breast cancer (14). Excessive expression of EZH2 can result through loss of EZH2-targeting miRNAs (e.g. miR-101 and miR-26A), increased E2F activity (loss of RB1 or CDKN2A), or amplification of EZH2 itself (15). The type of EZH2 dysregulation often correlates with the type of malignancy. For example, EZH2 overexpression is mainly found in solid tumors, whereas EZH2 activating mutations have been described in lymphomas. These result in an excess of H3K27me3 repressive marks impairing gene expression of tumor suppressor genes. EZH2-inactivating mutations, which lead to reduced H3K27me3 and derepression of genes contributing to leukemogenesis, have been found in myelodysplastic syndromes (3).

Increased expression of EZH2 has been associated with increased tumor cell proliferation and therefore worse survival (10, 14, 16, 17). The association between EZH2 and tumor aggressiveness has been confirmed in several tumor types (13, 18, 19) and resulted in the consideration of EZH2 as an important therapeutic target. The prognostic value of EZH2 seems to be clear but its predictive value was yet unknown. We described the association between high EZH2 levels and a poor outcome to tamoxifen therapy. A possible explanation can be the derived finding that less expression of EZH2 associates with a higher expression of ER which is the treatment target of tamoxifen. Based on cell line model studies, we proposed ER as a target of EZH2 where silencing of EZH2 would result in less H3K27me3 repressive marks on the promoter region of ER and as a consequence increased ER expression. There might be a role for Repressor of Estrogen receptor activity (REA) since this is a binding partner of EZH2 and binds the ER. It thus acts as a corepressor in estrogen-dependent transcription and regulates the transcription of estrogen-dependent genes. In line with our findings it has been shown that silencing of REA results in an increase of ER-dependent transcription (20).

EZH2 thus seems to be involved in endocrine therapy resistance and strategies reducing its expression might prevent or postpone therapy resistance. The development of targeted EZH2-inhibitors is therefore of great interest. It has been shown that two microRNAs (miRs) are able to regulate EZH2 expression in different tissues, i.e. miR-26a and miR-101 (21, 22). miRs are small non-protein coding RNAs that target coding mRNAs to repress translation or induce degradation of their target mRNAs. miR-26a has been found to be significantly active in lymph node negative disease suggesting its role as suppressor of lymph node invasion and is therefore associated with a better survival (23). This is in line with our findings that high miR-26a is associated with clinical benefit and favorable PFS in a group of MBC patients treated with first-line tamoxifen therapy (Chapter 3, this thesis). The overlapping genes between miR-26a and EZH2 were CCNE1 and CDC2, both involved in the cell cycle regulation and associated with shorter PFS when highly expressed. Moreover, miR-26a inhibits the epithelial-to-mesenchymal (EMT) process by repressing EZH2 (24). It might therefore be interesting to target EZH2 through upregulation of miR-26a through nanoparticles therapy. In this way, therapy resistance can possibly overcome while the development of metastases through inhibition of EMT gets suppressed.

One of the best known used EZH2 inhibitors is 3-dezaneplanocin-A (DZNep) which is a hydrolase inhibitor that targets S-adenosyl-L-homocysteine hydrolase (SAH). DZNep leads to an increase of SAH levels and thus to a repression of the activity of S-adenosyl-L-methionine (SAM)-dependent histone lysine methyltransferase activity such as EZH2 (25). The effect of DZNep in inhibiting histone methylation is not specific to EZH2 but has shown to induce antitumor activity in multiple cancer types and can at least be partly due to inhibition of PRC2 and removal of H3K27me3 marks (26). However, it has been shown that DZNep can also inhibit the active histone mark H3K4me3 (27). The lack of specificity of DZNep together with its short plasma half-life, its unknown effect on global methylation status and poor toxicity profile does not make it an attractive agent to introduce into the clinic. The development of more selective EZH2 inhibitors was therefore of great interest and has identified several potent inhibitors. In 2012, EPZ005687 has shown to be a potent inhibitor of EZH2 with a greater than 500-fold selectivity against 15 other protein methyltransferases and has 50-fold selectivity against the closely related enzyme EZH1 (28). EPZ005687 has exerted inhibition of H3K27me3 in EZH2-wild type and Y641- and A677-mutant lymphoma cells as well as in cell lines of breast and prostate cancer (25, 28).

An even more selective EZH2-inhibtor is GSK126 that has shown to be more than 1,000-fold selective for EZH2 versus 20 other human methyltransferases and 150-fold as compared to EZH1. GSK126 has shown to induce a 50% loss of H3K27me3 in both EZH2 wild-type and mutant lymphoma cell lines and did not affect PRC2 components in contrast to DZNep. Moreover, it has shown to be effective and well tolerated in an animal model (29). During the development of EPZ005687 and GSK126, a third selective SAM-competitive inhibitor, El1, was developed and showed to be highly selective (30). The discoveries of these agents have been an important development in the field of

epigenetic therapy. However, these compounds require frequent injection and are therefore less useful in the clinic. Hence UNC1999 has been synthesized as the first orally bioavailable inhibitor and showed to be highly selective for wild-type EZH2 and Y641 mutant as well as EZH1 (31).

The first EZH2-inhibitor that has led to phase I/II clinical trial is EPZ-6438 (tazemetostat). In this trial, 45 patients were included of whom 26 had relapsed or refractory solid tumors and 19 had lymphoma. There was an acceptable safety profile and preliminary results of the 15 tested patients with non-Hodgkin lymphoma showed partial or complete response in 9 of them (http://www.epizyme.com/wp-content/uploads/2015/07/ICML-Slides-Presented-062015-v2.pdf).

FUTURE PERSPECTIVES ON ER, EZH2, AND ENDOCRINE THERAPY

Although the prognostic value of EZH2 has already been widely investigated and recognized, the predictive value has been hardly studied. Since EZH2 has been shown to be highly expressed in more aggressive tumor types, its determination in clinical practice could be of use in choosing the right treatment strategy. Additionally, in this thesis, we have demonstrated that the expression of EZH2 is associated with outcome to both tamoxifen and AI therapy which underscores its predictive value. Before EZH2 can be introduced as a valuable marker in the clinic, prospective clinical trials should be conducted in order to identify subsets of patients that will benefit from a certain treatment based on their EZH2 expression levels. At first, it needs to be clarified if EZH2 expression can be measured in a standardized matter without much variability. Next, it would be interesting to determine if EZH2 can be adequately measured in both primary tumors and circulating biomarkers. At last, it needs to be further clarified if EZH2 itself can be used as a possible treatment target with an acceptable toxicity profile. Until now, the results of EZH2 inhibition are promising. It might be interesting to continue clinical trials specifically focused on the downstream effects of EZH2 inhibition. If, like in our cell line studies, it shows to upregulate or reinduce ER expression in patients with breast cancer, EZH2 inhibition could be a solution to endocrine therapy resistance. Nowadays, loss of ER expression due to clonal selection of ER negative cells or transcriptional repression of ER gene expression is one of the most known causes of resistance (32-34) and it has been shown that demethylating agents or histone deacetylase inhibitors can reactivate ER expression when its promotor has been methylated leading to renewed sensitivity to endocrine therapies (35, 36). Although these drugs have shown advantages in hematological disorders, challenges remain in the use of these compounds due to their instability in aqueous solutions and their rapid inactivation (37). In the area of solid malignancies, no epigenetic agents have yet been introduced into clinical practice but several trials have shown potential clinical benefit, e.g. a double-blind phase II study of exemestane with the HDAC inhibitor entinostat versus exemestane alone in 130 patients with ER-positive MBC who had previously progressed on an non-steroidal AI in which modest improvement in PFS was shown but an impressive difference in overall

survival was seen, indicating that epigenetic therapy may improve long-term outcomes for patients (38).

Although EZH2 inhibition seems to be promising to overcome endocrine resistance, it is known that genomic changes and clonal expansion of rare mutant clones occur over time and under treatment pressure, which may also yield endocrine resistance. Induction of different mutational profiles during therapy with EZH2 inhibition is therefore very well possible. For example, *ESR1* mutations are frequently acquired during the process of endocrine therapy resistance, especially during treatment with estrogen deprivation therapy like aromatase inhibitors (39, 40). These mutations are clustered in the ligand-binding domain (LBD) of the ER and induce constitutive ligand-independent ER activity that promotes tumor growth, contributing to the development of resistance (41-43). It has been shown that the mutant transcriptional activity can partly be reduced through higher doses of tamoxifen and fulvestrant (43) which might explain the finding that a higher dose of fulvestrant improved PFS and OS in a phase III trial including patients with ER-positive advanced breast cancer (44, 45). In contrast, it has been shown that patients with detected *ESR1* mutations in circulating tumor DNA (ctDNA) do not have a differential PFS with fulvestrant treatment compared with patients without *ESR1* mutations. However, a substantial majority of the *ESR1* mutations show a persistent decrease in allele frequency over time during treatment with fulvestrant or remain free of detectable plasma *ESR1* mutations (40). Presumably, selective estrogen receptor degraders (SERD) like fulvestrant are able to both degrade wild type and mutant ER.

ER, PI3K PATHWAY, AND ENDOCRINE THERAPY

New therapeutic strategies have been developed due to the expansion in knowledge on the molecular biology of both primary and secondary resistance. The PI3K/AKT/mTOR pathway has been shown to be one of the activated key pathways during endocrine therapy resistance (46, 47). Preclinical data provided evidence for combining mTOR inhibition and endocrine therapy due to its synergistic effect on inhibition of proliferation (48). Two landmark clinical trials confirmed these data. The phase II TAMRAD study in which tamoxifen was combined with everolimus showed an improvement in PFS and OS compared to tamoxifen alone in patients with MBC that had progressed during or after non-steroidal AI therapy (49). The BOLERO-2 study largely confirmed these findings but compared everolimus with exemestane to exemestane alone in MBC patients who failed previous treatment with non-steroidal AI therapy. The observed statistically prolonged PFS has led to the new standard of care for the treatment of endocrine-resistant postmenopausal MBC (50, 51). However, the combination therapy has recently shown not to significantly improve OS (52). Several other agents that target this pathway upstream of mTOR are currently under investigation and have shown promising results (53, 54).

Interestingly, treatment with agents targeting the PI3K/AKT/mTOR pathway only seems to be effective when secondary endocrine resistance has been developed as the HORIZON trial showed no improvement in PFS when endocrine-naïve patients were treated with letrozole and temsirolimus (mTOR antagonist) compared to letrozole alone which underscores the existence of more treatment options after failure of first-line AI therapy (55).

Preclinical studies resulted in evidence for increased cross-talk between growth factor receptor signaling pathways and ER at the time of relapse on endocrine therapy, providing a rationale for targeting both (56, 57). Although there are no conclusive clinical data to support the combination of EGFR-inhibition and endocrine therapy as first-line therapy, several clinical studies have shown improvement in PFS when ER+ HER2+ tumors are concurrently treated with anti-HER2 and endocrine therapy (54).
However, blockade of the HER2 pathway in ER+ HER2- MBC patients did not show an improvement in PFS thus fails to delay resistance (58). The combined therapy in these patients could probably become effective once resistance against endocrine therapy has developed, presumably due to induction of HER2 expression, as supported by xenograft studies (57).

Inhibition of the cell cycle forms a new therapeutic strategy since dysregulation of the cyclin-D-CDK4/6-INK4-Rb pathway is frequently observed in cancer and contributes to continuous growth. However, a major concern is that CDKs play an important role in the proliferation of both normal cells as well as cancer cells which creates a narrow therapeutic window. CDK4/6 activity plays key role in cell proliferation and thus forms an attractive therapeutic strategy. Palbociclib, an oral CDK4/6 inhibitor, prevents cellular DNA synthesis by blocking cell cycle progression from G1 to S phase. The combination of palbociclib and letrozole has shown an impressive and significantly improved PFS compared to letrozole alone in patients with ER+ MBC and has led to an accelerated FDA-approval (59). This approach seems also be attractive in endocrine resistant disease considering the improved PFS as shown in a recent study that compared palbociclib and fulvestrant with fulvestrant alone in patients with ER+ MBC that had progressive disease during prior endocrine therapy (60).
It would be interesting to combine a CDK4/6 inhibitor with an EZH2 inhibitor since EZH2 is a downstream target of CDK6 and inhibition of CDK6 has shown to downregulate the protein expression levels of both pRB and EZH2 (61). Combining both inhibitors could possibly lead to a synergistic effect on inhibition of cell growth. The combination of CDK4/6 inhibitors and PI3K inhibitors has already proven to be effective *in vitro*. In breast cancer cell lines where PI3K inhibition alone was relatively ineffective, the addition of a CDK4/6 inhibitor demonstrated a synergistic interaction in suppressing cell proliferation by induction of cell cycle arrest rather than apoptosis (62).

FUTURE PERSPECTIVES ON CIRCULATING TUMOR CELLS (CTCS) AND CTDNA

It has been shown that mutational profiles and *ESR1* mutations can extensively vary within the primary tumor of a patient but also between different metastatic sites within a patient (63, 64). It is thought that one of the major reasons for the current failure of cancer treatments is the inability to accurately monitor this spatial and temporal heterogeneity during tumor evolution. New technological advances using circulating biomarkers have been developed which have shown to be able to capture, amongst others, ESR mutations, leading to early treatment changes (65).

Until the development of circulating biomarkers it was necessary to take multiple biopsies in order to adequately monitor disease. However, this is an invasive and sometimes risky procedure and not always feasible. Moreover, the heterogeneity within a tumor harboring different mutational profiles requiring other treatment decisions can easily be missed. Hence, the characterization of circulating biomarkers poses an attractive alternative. Both CTCs and ctDNA are thought to originate from either the primary tumor or metastases and can be obtained through venipunctures. This way, analyses of CTCs and ctDNA are considered a real-time 'liquid biopsy'.

THE DETECTION AND USE OF CTCS

The detection of CTCs is challenging with a median CTC count of 3 – 5 per 7.5 mL of blood in MBC patients (66). Over the years many CTC detection assays have been developed but the CellSearch system is yet the only FDA-cleared system. It isolates CTCs using surface protein expression of the epithelial marker EpCAM. Major drawback is that CTCs undergo epithelial-to-mesenchymal transition (EMT) that results in reduced expression of epithelial markers which might lead to false-negative findings when using techniques like CellSearch (67). During the process of EMT, different subsets of CTCs show a range of phenotypes. In order to isolate these CTCs a large cocktail of both epithelial and mesenchymal markers would be necessary increasing the risk of isolating individual blood cells expressing at least one of these markers resulting in false-positive results (68, 69). This limitation can be overcome by using actin bundling protein plastin 3 which is not expressed by blood cells and does not to get downregulated during EMT (70). In breast cancer, this marker has recently been identified as a biomarker to identify patients at risk of recurrence or with a poor prognosis (71).

The first study that reported the prognostic value of CTC count in MBC dates from 2004 (72) which has been confirmed in a pooled analysis ten years later in which CTC count showed to be a dynamic prognostic marker of PFS and OS (73).
Interestingly, CTCs can also be detected in patients presenting with primary breast cancer without overt metastatic disease and appear to be an independent predictor of poor disease-free, overall, breast cancer-specific, and distant disease-free survival (74). The reported rates might be an underestimation since this study used the CellSearch system in which the more aggressive CTC types that have undergone EMT are possibly missed.

There already have been performed several CTC-driven clinical trials of which the SWOG S0500 trial is the most widely known. In this trial patients with persistently elevated CTCs (≥5 CTCs/7.5 mL) after one cycle of chemotherapy were randomized between continuation of the first-line chemotherapy or early switch to another chemotherapy regimen. The study confirmed the prognostic significance of CTC measurements since patients who had a decline in CTC number after the first cycle of chemotherapy had a longer OS compared to patients whose baseline CTCs did not decline. However, an early switch to an alternative chemotherapy regimen failed to improve OS or PFS (75). The trial however included a heterogeneous population of MBC patients that could have received endocrine or biologic therapies before inclusion. Moreover, CTC change was only assessed as a binary categorical variable which implies that major changes in CTC numbers were not scored unless they crossed the threshold of 5 CTCs/7.5 mL at the second measurement. Next, it is uncommon for breast cancer that has resistance to one chemotherapy regimen to exhibit a high degree of sensitivity to a randomly selected alternative chemotherapy.
Future clinical trials, taking into account the missed aspects in SWOG S0500, should further investigate the potential contribution CTCs can have in clinical decision making.

Besides detection and enumeration of CTCs the major clinical challenge encompasses molecular characterization of CTCs in order to base clinical decision making on the current tumor characteristics. CTCs can be characterized on different levels of which protein level might be the most interesting as ER is a key target in breast cancer. It has been demonstrated that ER+ breast tumors can frequently harbor ER- CTCs which may reflect an escape mechanism to endocrine therapy (76). *ESR1* mutations were not found in this study when performing single cell analysis on the CTCs drawn from ER+ MBC untreated patients which is consistent with the finding that these mutations only occur after endocrine treatment failure.
The HER2 oncogene has also shown to have a discrepant status between primary tumor and CTCs in up to 30% of cases (77). This is particular interesting in patients with HER2-negative primary tumors and HER2+ CTCs since it could lead to more extensive treatment options if these patients respond to HER2-targeting therapies.
More recently, immune checkpoint regulators such as PD-1/PD-L1 have become new therapeutic targets which is in line with the lack of immunologic control as one of

the emerging hallmarks of cancer (78). It has shown promising results in many tumor types including advanced melanoma and non-small cell lung cancer (NSCLC) patients. Recently, it has been demonstrated that PD-L1 is expressed on CTCs of ER+ HER2- MBC patients (79) which might form a rationale to select these patients for anti-PD-1/PD-L1 therapy.

In addition to determining protein expression in CTCs of breast cancer patients, mRNA analyses can also be performed (80) but have not yet shown to lead to treatment changes. Our 8-gene CTC profile is the first study that has generated a unique mRNA CTC-based gene panel that is able to distinguish good and poor responders to first-line AI therapy which could impact clinical decision making. This way, CTC characterization can be used as a predictor for response to endocrine therapy which may better reflect the current disease burden and therapy sensitivity of an MBC patient in order to overcome over- or undertreatment. Before it can be implemented in clinical practice, extensive prospective validation is warranted. This can be difficult since it should be taken into account that this profile has been based on CTCs captured with the CellSearch system potentially missing out on the CTCs that have undergone EMT. Moreover, the CTC profile has been measured in the total amount of detected CTCs thus heterogeneity between CTCs has not been taken into account. Although single cell analyses of CTCs might therefore be of more interest, CTC characterization of the total amount of CTCs is possibly an adequate reflection of the overall tumor burden and, moreover, easier to implement in clinical practice.

On DNA level, both *TP53* and *PIK3CA* mutations have been detected in CTCs (81, 82). This could lead to treatment changes since it has been reported that PI3K hyperactivity contributes to a lower response to trastuzumab and lapatinib in patients with HER2+ tumors as well as to resistance to anti-estrogen therapies (83, 84). The combination of PI3K-inhibition and antiestrogen therapy has shown to induce regression of ER+/*PIK3CA* mutant breast tumors. However, there has been demonstrated a strong heterogeneity in the mutational status among CTCs from individual patients making it difficult to come to an appropriate treatment strategy (82).

CIRCULATING TUMOR DNA (CTDNA)

Another circulating biomarker that can serve as a new target for antitumor therapy is ctDNA, i.e. DNA that is shed by damaged, apoptotic, and necrotic tumor cells into the blood. It can be released from primary tumors, CTCs, micrometastasis, or overt metastases (85, 86). Since ctDNA has a half-life of less than 2 hours it is thought to be a good representation of the present tumor load (87). Levels of ctDNA vary a lot between patients and can be hard to detect amongst background levels of circulating wild-type DNA, especially for small tumors in early stages. The development of sensitive techniques such as BEAMing and digital PCR made it possible to detect and quantify minute amounts of DNA. False positives are expected to be rare because ctDNA is defined by mutations and other genomic changes that are hallmarks of cancer cells (87).

In a large study that included multiple cancer types it was shown that ctDNA can be detected in most solid tumors outside the brain although the fraction varied with tumor type and among patients with the same tumor type. ctDNA can also be detected in patients with localized disease but in a lower fraction than patients with advanced disease (88, 89). In patients with localized disease, the level of baseline ctDNA showed not to be predictive for disease-free survival but ctDNA detection in a postoperative sample was highly predictive of early relapse even so was ctDNA detection in serial samples. The presence of ctDNA seems to identify relapse before clinical relapse on conventional imaging (89). Although results are fairly promising, it needs to be taken into account that this study included only patients treated with neoadjuvant chemotherapy and sample size was quite small.

ctDNA might be more sensitive than CTCs as ctDNA fragments outnumbered CTCs in the previous described study. All patients with CTCs had ctDNA whereas not all patients with ctDNA had a trace of CTCs (88). However, most of the included patients in this subanalysis had low stage cancer whereas CTCs are thought to be mainly present in advanced disease whereas ctDNA is more often detected in both localized and advanced disease.

Just as in CTCs, the major clinical challenge in ctDNA is not to detect its presence but to detect the molecular characteristics it harbors. At present, several genomic alterations can be detected in ctDNA of various tumor types (90). For example, it has been shown to be possible to detect *PIK3CA* mutations in ctDNA of primary breast cancer patients. The amount of detected *PIK3CA* mutated ctDNA was related to a shorter recurrence-free survival and OS (91).
In a study with patients with MBC, ctDNA was collected and somatic genomic alterations were identified in *TP53* and *PIK3CA*. In this study, ctDNA levels showed a greater dynamic range, and greater correlation with changes in tumor burden, than did CA15-3 or CTCs detected by the CellSearch system (92). However, this study only investigated 52 patients.
Next, *ESR1* mutations can robustly be identified in ctDNA of ER+ MBC patients. *ESR1* mutations are rarely acquired during adjuvant AI therapy but commonly selected during first-line AI therapy and can occur in a polyclonal manner (39, 93). Since these mutations predict for resistance to subsequent AI therapy, detected *ESR1* mutations in ctDNA before disease progression occurs can lead to an early change in therapeutic options with potential benefit to patients (94).

It will be crucial to select the correct time point for ctDNA screening in order to detect those ctDNA variants that are derived from the resistant tumor cell clones. Parallel determination of viable CTCs might provide the required additional information to tailor the therapy to the need of the cancer patient (86). Moreover, before ctDNA analysis can be implemented in the management of cancer patients, tests need to be standardized to make them as reliable and reproducible as CTC tests already are.

FUTURE PERSPECTIVES ON MEDICAL ONCOLOGY

The development of new endocrine and targeted therapies has prolonged the clinical benefit for women with ER+ MBC in such manner that the time that cytotoxic chemotherapy is required has been extensively delayed. However, targeting a solitary signaling pathway will not reverse all endocrine therapy resistance mechanisms. Blockade of one signaling pathway will promote the development of compensatory escape mechanisms which forms the rationale for the development of combination therapies that target key signaling pathways in parallel (horizontal blockade) or in series (vertical blockade) (54). To select the most appropriate population for each form of therapy it is probably useful to find the relevant biomarker through real-time biopsy by using a circulating biomarker as the best representative for metastatic disease in order to maximize the likelihood of success in preventing, postponing or reversing endocrine therapy resistance.
Liquid biopsies can be used to monitor patients being treated with targeted agents to provide longitudinal monitoring of genomic alterations and check for the emergence of treatment-resistant clones over time. However, identifying tumor recurrence at an earlier time point does not improve clinical outcome if an effective therapy is not selected or available. Moreover, a risk is the detection of clinically irrelevant molecular changes due to the high sensitivity of the methods. Next, it might be difficult to discriminate between driver and passenger mutations. Large annotated datasets and bioinformatic tools next to sharing of clinical data will be needed to distinguish potentially important genomic aberrations from noise (95).

It is expected that in the near future of medical oncology new therapeutics will be developed especially targeting those mutations that will be discovered from circulating biomarker research and show to be involved in treatment resistance. It is therefore necessary to determine what level of alteration/mutation in a key driver is sufficient to initiate a switch in disease management before circulating biomarkers can be introduced in the clinic.

The future of medical oncology will eventually consist of precision medicine. The right treatment for a patient should be constantly tailored using both primary tumor and circulating biomarker information in which CTCs and ctDNA can both contribute in a different and complementary matter. Besides genomic data derived from both primary and circulating tumor parts, this thesis underscores the importance of epigenetics as has also recently been demonstrated (96). The role of epigenetics is not yet exactly known but its importance is recognized and has led to a broader range of targeted therapy. These agents warrant more research before implementation in the clinic but it is expected that they will contribute to a better survival, and hopefully quality of life, of MBC patients.

REFERENCES

1. Early Breast Cancer Trialists' Collaborative G. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet. 2005;365(9472):1687-717.
2. Jansen MP, Foekens JA, van Staveren IL, Dirkzwager-Kiel MM, Ritstier K, Look MP, et al. Molecular classification of tamoxifen-resistant breast carcinomas by gene expression profiling. J Clin Oncol. 2005;23(4):732-40.
3. Volkel P, Dupret B, Le Bourhis X, Angrand PO. Diverse involvement of EZH2 in cancer epigenetics. Am J Transl Res. 2015;7(2):175-93.
4. Bracken AP, Dietrich N, Pasini D, Hansen KH, Helin K. Genome-wide mapping of Polycomb target genes unravels their roles in cell fate transitions. Genes Dev. 2006;20(9):1123-36.
5. Cao R, Wang L, Wang H, Xia L, Erdjument-Bromage H, Tempst P, et al. Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science. 2002;298(5595):1039-43.
6. Schuettengruber B, Chourrout D, Vervoort M, Leblanc B, Cavalli G. Genome regulation by polycomb and trithorax proteins. Cell. 2007;128(4):735-45.
7. Varambally S, Dhanasekaran SM, Zhou M, Barrette TR, Kumar-Sinha C, Sanda MG, et al. The polycomb group protein EZH2 is involved in progression of prostate cancer. Nature. 2002;419(6907):624-9.
8. Raaphorst FM, van Kemenade FJ, Blokzijl T, Fieret E, Hamer KM, Satijn DP, et al. Coexpression of BMI-1 and EZH2 polycomb group genes in Reed-Sternberg cells of Hodgkin's disease. Am J Pathol. 2000;157(3):709-15.
9. Visser HP, Gunster MJ, Kluin-Nelemans HC, Manders EM, Raaphorst FM, Meijer CJ, et al. The Polycomb group protein EZH2 is upregulated in proliferating, cultured human mantle cell lymphoma. Br J Haematol. 2001;112(4):950-8.
10. Bachmann IM, Halvorsen OJ, Collett K, Stefansson IM, Straume O, Haukaas SA, et al. EZH2 expression is associated with high proliferation rate and aggressive tumor subgroups in cutaneous melanoma and cancers of the endometrium, prostate, and breast. J Clin Oncol. 2006;24(2):268-73.
11. Raman JD, Mongan NP, Tickoo SK, Boorjian SA, Scherr DS, Gudas LJ. Increased expression of the polycomb group gene, EZH2, in transitional cell carcinoma of the bladder. Clin Cancer Res. 2005;11(24 Pt 1):8570-6.
12. Weikert S, Christoph F, Kollermann J, Muller M, Schrader M, Miller K, et al. Expression levels of the EZH2 polycomb transcriptional repressor correlate with aggressiveness and invasive potential of bladder carcinomas. Int J Mol Med. 2005;16(2):349-53.
13. Li H, Cai Q, Godwin AK, Zhang R. Enhancer of zeste homolog 2 promotes the proliferation and invasion of epithelial ovarian cancer cells. Mol Cancer Res. 2010;8(12):1610-8.
14. Kleer CG, Cao Q, Varambally S, Shen R, Ota I, Tomlins SA, et al. EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells. Proc Natl Acad Sci U S A. 2003;100(20):11606-11.
15. McCabe MT, Creasy CL. EZH2 as a potential target in cancer therapy. Epigenomics. 2014;6(3):341-51.
16. Collett K, Eide GE, Arnes J, Stefansson IM, Eide J, Braaten A, et al. Expression of enhancer

of zeste homologue 2 is significantly associated with increased tumor cell proliferation and is a marker of aggressive breast cancer. Clin Cancer Res. 2006;12(4):1168-74.
17. Raaphorst FM, Meijer CJ, Fieret E, Blokzijl T, Mommers E, Buerger H, et al. Poorly differentiated breast carcinoma is associated with increased expression of the human polycomb group EZH2 gene. Neoplasia. 2003;5(6):481-8.
18. Eskander RN, Ji T, Huynh B, Wardeh R, Randall LM, Hoang B. Inhibition of enhancer of zeste homolog 2 (EZH2) expression is associated with decreased tumor cell proliferation, migration, and invasion in endometrial cancer cell lines. Int J Gynecol Cancer. 2013;23(6):997-1005.
19. Ougolkov AV, Bilim VN, Billadeau DD. Regulation of pancreatic tumor cell proliferation and chemoresistance by the histone methyltransferase enhancer of zeste homologue 2. Clin Cancer Res. 2008;14(21):6790-6.
20. Hwang C, Giri VN, Wilkinson JC, Wright CW, Wilkinson AS, Cooney KA, et al. EZH2 regulates the transcription of estrogen-responsive genes through association with REA, an estrogen receptor corepressor. Breast Cancer Res Treat. 2008;107(2):235-42.
21. Varambally S, Cao Q, Mani RS, Shankar S, Wang X, Ateeq B, et al. Genomic loss of microRNA-101 leads to overexpression of histone methyltransferase EZH2 in cancer. Science. 2008;322(5908):1695-9.
22. Wong CF, Tellam RL. MicroRNA-26a targets the histone methyltransferase Enhancer of Zeste homolog 2 during myogenesis. J Biol Chem. 2008;283(15):9836-43.
23. Smeets A, Daemen A, Vanden Bempt I, Gevaert O, Claes B, Wildiers H, et al. Prediction of lymph node involvement in breast cancer from primary tumor tissue using gene expression profiling and miRNAs. Breast Cancer Res Treat. 2011;129(3):767-76.
24. Ma DN, Chai ZT, Zhu XD, Zhang N, Zhan DH, Ye BG, et al. MicroRNA-26a suppresses epithelial-mesenchymal transition in human hepatocellular carcinoma by repressing enhancer of zeste homolog 2. J Hematol Oncol. 2016;9(1):1.
25. Kim KH, Roberts CW. Targeting EZH2 in cancer. Nat Med. 2016;22(2):128-34.
26. Tan J, Yang X, Zhuang L, Jiang X, Chen W, Lee PL, et al. Pharmacologic disruption of Polycomb-repressive complex 2-mediated gene repression selectively induces apoptosis in cancer cells. Genes Dev. 2007;21(9):1050-63.
27. Miranda TB, Cortez CC, Yoo CB, Liang G, Abe M, Kelly TK, et al. DZNep is a global histone methylation inhibitor that reactivates developmental genes not silenced by DNA methylation. Mol Cancer Ther. 2009;8(6):1579-88.
28. Knutson SK, Wigle TJ, Warholic NM, Sneeringer CJ, Allain CJ, Klaus CR, et al. A selective inhibitor of EZH2 blocks H3K27 methylation and kills mutant lymphoma cells. Nat Chem Biol. 2012;8(11):890-6.
29. McCabe MT, Ott HM, Ganji G, Korenchuk S, Thompson C, Van Aller GS, et al. EZH2 inhibition as a therapeutic strategy for lymphoma with EZH2-activating mutations. Nature. 2012;492(7427):108-12.
30. Qi W, Chan H, Teng L, Li L, Chuai S, Zhang R, et al. Selective inhibition of Ezh2 by a small molecule inhibitor blocks tumor cells proliferation. Proc Natl Acad Sci U S A. 2012;109(52):21360-5.
31. Konze KD, Ma A, Li F, Barsyte-Lovejoy D, Parton T, Macnevin CJ, et al. An orally

bioavailable chemical probe of the Lysine Methyltransferases EZH2 and EZH1. ACS Chem Biol. 2013;8(6):1324-34.

32. Amir E, Miller N, Geddie W, Freedman O, Kassam F, Simmons C, et al. Prospective study evaluating the impact of tissue confirmation of metastatic disease in patients with breast cancer. J Clin Oncol. 2012;30(6):587-92.
33. Gong Y, Han EY, Guo M, Pusztai L, Sneige N. Stability of estrogen receptor status in breast carcinoma: a comparison between primary and metastatic tumors with regard to disease course and intervening systemic therapy. Cancer. 2011;117(4):705-13.
34. Thompson AM, Jordan LB, Quinlan P, Anderson E, Skene A, Dewar JA, et al. Prospective comparison of switches in biomarker status between primary and recurrent breast cancer: the Breast Recurrence In Tissues Study (BRITS). Breast Cancer Res. 2010;12(6):R92.
35. Ferguson AT, Lapidus RG, Baylin SB, Davidson NE. Demethylation of the estrogen receptor gene in estrogen receptor-negative breast cancer cells can reactivate estrogen receptor gene expression. Cancer Res. 1995;55(11):2279-83.
36. Sharma D, Saxena NK, Davidson NE, Vertino PM. Restoration of tamoxifen sensitivity in estrogen receptor-negative breast cancer cells: tamoxifen-bound reactivated ER recruits distinctive corepressor complexes. Cancer Res. 2006;66(12):6370-8.
37. Katz TA, Huang Y, Davidson NE, Jankowitz RC. Epigenetic reprogramming in breast cancer: from new targets to new therapies. Ann Med. 2014;46(6):397-408.
38. Yardley DA, Ismail-Khan RR, Melichar B, Lichinitser M, Munster PN, Klein PM, et al. Randomized phase II, double-blind, placebo-controlled study of exemestane with or without entinostat in postmenopausal women with locally recurrent or metastatic estrogen receptor-positive breast cancer progressing on treatment with a nonsteroidal aromatase inhibitor. J Clin Oncol. 2013;31(17):2128-35.
39. Schiavon G, Hrebien S, Garcia-Murillas I, Cutts RJ, Pearson A, Tarazona N, et al. Analysis of ESR1 mutation in circulating tumor DNA demonstrates evolution during therapy for metastatic breast cancer. Sci Transl Med. 2015;7(313):313ra182.
40. Spoerke JM, Gendreau S, Walter K, Qiu J, Wilson TR, Savage H, et al. Heterogeneity and clinical significance of ESR1 mutations in ER-positive metastatic breast cancer patients receiving fulvestrant. Nat Commun. 2016;7:11579.
41. Jeselsohn R, Yelensky R, Buchwalter G, Frampton G, Meric-Bernstam F, Gonzalez-Angulo AM, et al. Emergence of constitutively active estrogen receptor-alpha mutations in pretreated advanced estrogen receptor-positive breast cancer. Clin Cancer Res. 2014;20(7):1757-67.
42. Robinson DR, Wu YM, Vats P, Su F, Lonigro RJ, Cao X, et al. Activating ESR1 mutations in hormone-resistant metastatic breast cancer. Nat Genet. 2013;45(12):1446-51.
43. Toy W, Shen Y, Won H, Green B, Sakr RA, Will M, et al. ESR1 ligand-binding domain mutations in hormone-resistant breast cancer. Nat Genet. 2013;45(12):1439-45.
44. Di Leo A, Jerusalem G, Petruzelka L, Torres R, Bondarenko IN, Khasanov R, et al. Results of the CONFIRM phase III trial comparing fulvestrant 250 mg with fulvestrant 500 mg in postmenopausal women with estrogen receptor-positive advanced breast cancer. J Clin

Oncol. 2010;28(30):4594-600.
45. Di Leo A, Jerusalem G, Petruzelka L, Torres R, Bondarenko IN, Khasanov R, et al. Final overall survival: fulvestrant 500 mg vs 250 mg in the randomized CONFIRM trial. J Natl Cancer Inst. 2014;106(1):djt337.
46. Cavazzoni A, Bonelli MA, Fumarola C, La Monica S, Airoud K, Bertoni R, et al. Overcoming acquired resistance to letrozole by targeting the PI3K/AKT/mTOR pathway in breast cancer cell clones. Cancer Lett. 2012;323(1):77-87.
47. Miller TW, Balko JM, Arteaga CL. Phosphatidylinositol 3-kinase and antiestrogen resistance in breast cancer. J Clin Oncol. 2011;29(33):4452-61.
48. Boulay A, Rudloff J, Ye J, Zumstein-Mecker S, O'Reilly T, Evans DB, et al. Dual inhibition of mTOR and estrogen receptor signaling in vitro induces cell death in models of breast cancer. Clin Cancer Res. 2005;11(14):5319-28.
49. Bachelot T, Bourgier C, Cropet C, Ray-Coquard I, Ferrero JM, Freyer G, et al. Randomized phase II trial of everolimus in combination with tamoxifen in patients with hormone receptor-positive, human epidermal growth factor receptor 2-negative metastatic breast cancer with prior exposure to aromatase inhibitors: a GINECO study. J Clin Oncol. 2012;30(22):2718-24.
50. Yardley DA, Noguchi S, Pritchard KI, Burris HA, 3rd, Baselga J, Gnant M, et al. Everolimus plus exemestane in postmenopausal patients with HR(+) breast cancer: BOLERO-2 final progression-free survival analysis. Adv Ther. 2013;30(10):870-84.
51. Baselga J, Campone M, Piccart M, Burris HA, 3rd, Rugo HS, Sahmoud T, et al. Everolimus in postmenopausal hormone-receptor-positive advanced breast cancer. N Engl J Med. 2012;366(6):520-9.
52. Piccart M, Hortobagyi GN, Campone M, Pritchard KI, Lebrun F, Ito Y, et al. Everolimus plus exemestane for hormone-receptor-positive, human epidermal growth factor receptor-2-negative advanced breast cancer: overall survival results from BOLERO-2dagger. Ann Oncol. 2014;25(12):2357-62.
53. Ciruelos Gil EM. Targeting the PI3K/AKT/mTOR pathway in estrogen receptor-positive breast cancer. Cancer Treat Rev. 2014;40(7):862-71.
54. Johnston SR. Enhancing Endocrine Therapy for Hormone Receptor-Positive Advanced Breast Cancer: Cotargeting Signaling Pathways. J Natl Cancer Inst. 2015;107(10).
55. Wolff AC, Lazar AA, Bondarenko I, Garin AM, Brincat S, Chow L, et al. Randomized phase III placebo-controlled trial of letrozole plus oral temsirolimus as first-line endocrine therapy in postmenopausal women with locally advanced or metastatic breast cancer. J Clin Oncol. 2013;31(2):195-202.
56. Kurokawa H, Arteaga CL. Inhibition of erbB receptor (HER) tyrosine kinases as a strategy to abrogate antiestrogen resistance in human breast cancer. Clin Cancer Res. 2001;7(12 Suppl):4436s-42s; discussion 11s-12s.
57. Sabnis G, Schayowitz A, Goloubeva O, Macedo L, Brodie A. Trastuzumab reverses letrozole resistance and amplifies the sensitivity of breast cancer cells to estrogen. Cancer Res. 2009;69(4):1416-28.
58. Johnston S, Pippen J, Jr., Pivot X, Lichinitser M, Sadeghi S, Dieras V, et al. Lapatinib combined with letrozole versus letrozole and placebo as first-line therapy for postmenopausal hormone receptor-positive metastatic breast cancer. J Clin Oncol.

2009;27(33):5538-46.
59. Finn RS, Crown JP, Lang I, Boer K, Bondarenko IM, Kulyk SO, et al. The cyclin-dependent kinase 4/6 inhibitor palbociclib in combination with letrozole versus letrozole alone as first-line treatment of oestrogen receptor-positive, HER2-negative, advanced breast cancer (PALOMA-1/TRIO-18): a randomised phase 2 study. Lancet Oncol. 2015;16(1):25-35.
60. Turner NC, Ro J, Andre F, Loi S, Verma S, Iwata H, et al. Palbociclib in Hormone-Receptor-Positive Advanced Breast Cancer. N Engl J Med. 2015;373(3):209-19.
61. van der Linden MH, Willekes M, van Roon E, Seslija L, Schneider P, Pieters R, et al. MLL fusion-driven activation of CDK6 potentiates proliferation in MLL-rearranged infant ALL. Cell Cycle. 2014;13(5):834-44.
62. Vora SR, Juric D, Kim N, Mino-Kenudson M, Huynh T, Costa C, et al. CDK 4/6 inhibitors sensitize PIK3CA mutant breast cancer to PI3K inhibitors. Cancer Cell. 2014;26(1):136-49.
63. Ng CK, Schultheis AM, Bidard FC, Weigelt B, Reis-Filho JS. Breast cancer genomics from microarrays to massively parallel sequencing: paradigms and new insights. J Natl Cancer Inst. 2015;107(5).
64. Yates LR, Campbell PJ. Evolution of the cancer genome. Nat Rev Genet. 2012;13(11):795-806.
65. Sefrioui D, Perdrix A, Sarafan-Vasseur N, Dolfus C, Dujon A, Picquenot JM, et al. Short report: Monitoring ESR1 mutations by circulating tumor DNA in aromatase inhibitor resistant metastatic breast cancer. Int J Cancer. 2015;137(10):2513-9.
66. Allard WJ, Matera J, Miller MC, Repollet M, Connelly MC, Rao C, et al. Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. Clin Cancer Res. 2004;10(20):6897-904.
67. Rao CG, Chianese D, Doyle GV, Miller MC, Russell T, Sanders RA, Jr., et al. Expression of epithelial cell adhesion molecule in carcinoma cells present in blood and primary and metastatic tumors. Int J Oncol. 2005;27(1):49-57.
68. Yu M, Bardia A, Wittner BS, Stott SL, Smas ME, Ting DT, et al. Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. Science. 2013;339(6119):580-4.
69. Alix-Panabieres C, Pantel K. Challenges in circulating tumour cell research. Nat Rev Cancer. 2014;14(9):623-31.
70. Yokobori T, Iinuma H, Shimamura T, Imoto S, Sugimachi K, Ishii H, et al. Plastin3 is a novel marker for circulating tumor cells undergoing the epithelial-mesenchymal transition and is associated with colorectal cancer prognosis. Cancer Res. 2013;73(7):2059-69.
71. Ueo H, Sugimachi K, Gorges TM, Bartkowiak K, Yokobori T, Muller V, et al. Circulating tumour cell-derived plastin3 is a novel marker for predicting long-term prognosis in patients with breast cancer. Br J Cancer. 2015;112(9):1519-26.
72. Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med. 2004;351(8):781-91.
73. Bidard FC, Peeters DJ, Fehm T, Nole F, Gisbert-Criado R, Mavroudis D, et al. Clinical

validity of circulating tumour cells in patients with metastatic breast cancer: a pooled analysis of individual patient data. Lancet Oncol. 2014;15(4):406-14.
74. Janni WJ, Rack B, Terstappen LW, Pierga JY, Taran FA, Fehm T, et al. Pooled Analysis of the Prognostic Relevance of Circulating Tumor Cells in Primary Breast Cancer. Clin Cancer Res. 2016;22(10):2583-93.
75. Smerage JB, Barlow WE, Hortobagyi GN, Winer EP, Leyland-Jones B, Srkalovic G, et al. Circulating tumor cells and response to chemotherapy in metastatic breast cancer: SWOG S0500. J Clin Oncol. 2014;32(31):3483-9.
76. Babayan A, Hannemann J, Spotter J, Muller V, Pantel K, Joosse SA. Heterogeneity of estrogen receptor expression in circulating tumor cells from metastatic breast cancer patients. PLoS One. 2013;8(9):e75038.
77. Fehm T, Muller V, Aktas B, Janni W, Schneeweiss A, Stickeler E, et al. HER2 status of circulating tumor cells in patients with metastatic breast cancer: a prospective, multicenter trial. Breast Cancer Res Treat. 2010;124(2):403-12.
78. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646-74.
79. Mazel M, Jacot W, Pantel K, Bartkowiak K, Topart D, Cayrefourcq L, et al. Frequent expression of PD-L1 on circulating breast cancer cells. Mol Oncol. 2015;9(9):1773-82.
80. Sieuwerts AM, Mostert B, Bolt-de Vries J, Peeters D, de Jongh FE, Stouthard JM, et al. mRNA and microRNA expression profiles in circulating tumor cells and primary tumors of metastatic breast cancer patients. Clin Cancer Res. 2011;17(11):3600-18.
81. Fernandez SV, Bingham C, Fittipaldi P, Austin L, Palazzo J, Palmer G, et al. TP53 mutations detected in circulating tumor cells present in the blood of metastatic triple negative breast cancer patients. Breast Cancer Res. 2014;16(5):445.
82. Pestrin M, Salvianti F, Galardi F, De Luca F, Turner N, Malorni L, et al. Heterogeneity of PIK3CA mutational status at the single cell level in circulating tumor cells from metastatic breast cancer patients. Mol Oncol. 2015;9(4):749-57.
83. Berns K, Horlings HM, Hennessy BT, Madiredjo M, Hijmans EM, Beelen K, et al. A functional genetic approach identifies the PI3K pathway as a major determinant of trastuzumab resistance in breast cancer. Cancer Cell. 2007;12(4):395-402.
84. Eichhorn PJ, Gili M, Scaltriti M, Serra V, Guzman M, Nijkamp W, et al. Phosphatidylinositol 3-kinase hyperactivation results in lapatinib resistance that is reversed by the mTOR/phosphatidylinositol 3-kinase inhibitor NVP-BEZ235. Cancer Res. 2008;68(22):9221-30.
85. Spellman PT, Gray JW. Detecting cancer by monitoring circulating tumor DNA. Nat Med. 2014;20(5):474-5.
86. Alix-Panabieres C, Pantel K. Clinical Applications of Circulating Tumor Cells and Circulating Tumor DNA as Liquid Biopsy. Cancer Discov. 2016;6(5):479-91.
87. Yong E. Cancer biomarkers: Written in blood. Nature. 2014;511(7511):524-6.
88. Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. Sci Transl Med. 2014;6(224):224ra24.
89. Garcia-Murillas I, Schiavon G, Weigelt B, Ng C, Hrebien S, Cutts RJ, et al. Mutation tracking in circulating tumor DNA predicts relapse in early breast cancer. Sci Transl Med. 2015;7(302):302ra133.

90. Gingras I, Salgado R, Ignatiadis M. Liquid biopsy: will it be the 'magic tool' for monitoring response of solid tumors to anticancer therapies? Curr Opin Oncol. 2015;27(6):560-7.
91. Oshiro C, Kagara N, Naoi Y, Shimoda M, Shimomura A, Maruyama N, et al. PIK3CA mutations in serum DNA are predictive of recurrence in primary breast cancer patients. Breast Cancer Res Treat. 2015;150(2):299-307.
92. Dawson SJ, Tsui DW, Murtaza M, Biggs H, Rueda OM, Chin SF, et al. Analysis of circulating tumor DNA to monitor metastatic breast cancer. N Engl J Med. 2013;368(13):1199-209.
93. Wang P, Bahreini A, Gyanchandani R, Lucas PC, Hartmaier RJ, Watters RJ, et al. Sensitive Detection of Mono- and Polyclonal ESR1 Mutations in Primary Tumors, Metastatic Lesions, and Cell-Free DNA of Breast Cancer Patients. Clin Cancer Res. 2016;22(5):1130-7.
94. De Mattos-Arruda L, Caldas C. Cell-free circulating tumour DNA as a liquid biopsy in breast cancer. Mol Oncol. 2016;10(3):464-74.
95. Ignatiadis M, Lee M, Jeffrey SS. Circulating Tumor Cells and Circulating Tumor DNA: Challenges and Opportunities on the Path to Clinical Utility. Clin Cancer Res. 2015;21(21):4786-800.
96. Chen X, Hu H, He L, Yu X, Liu X, Zhong R, et al. A novel subtype classification and risk of breast cancer by histone modification profiling. Breast Cancer Res Treat. 2016;157(2):267-79.

CHAPTER 8

Summary
Samenvatting

SUMMARY

Over the past years, cancer has become the most common cause of death in The Netherlands. From all types of cancer, breast cancer is the most common cancer in females. Fortunately, the majority of patients presents with low stage breast cancer and has a relatively favorable prognosis. This prognosis is dependent on stage at presentation, which encompasses tumor size, lymph node status and presence or absence of distant metastases. Besides, the molecular classification is an important prognostic and predictive factor of which the estrogen receptor (ER), the progesterone receptor (PR), and HER2-neu are best known. The development of many therapies that target the ER have dramatically improved the prognosis of breast cancer and is considered as endocrine therapy. Tamoxifen, a selective estrogen receptor modulator, forms the mainstay of treatment for premenopausal women for more than 30 years. It competes with estradiol for the binding to ER and represses the transcriptional activity of ER by inducing a conformational change of the receptor.

An alternative strategy within the spectrum of endocrine therapy is the inhibitors of aromatase (AI), which are mainly used for postmenopausal women. AI therapy depletes serum estradiol levels by inhibiting the aromatase enzyme and therefore less growth factor is available for the receptor and consequently for the tumor.

In metastatic breast cancer (MBC), endocrine therapy is still the mainstay of treatment because of the favorable toxicity profile. Although many patients greatly benefit from endocrine therapies, it is known that approximately 30% of the MBC patients never respond due to *de novo* resistance. Besides, all initial responders eventually relapse. Therefore, MBC is still not a curable disease despite the current knowledge on the disease and the development of new therapies. Better understanding of the mechanisms involved in therapy resistance could lead to a change in treatment and thus possibly postpone the occurrence of resistance. The generation of biomarkers can lead to a better and more targeted individualized treatment. Therefore, as described in **chapter 1**, the goal of this thesis is to discover and understand (epigenetic) factors involved in endocrine therapy resistance. To reach this goal, cell line models were used next to both primary tumors and circulating tumor cells (CTCs) derived from patients.

Chapter 2 describes the finding of EZH2 being involved in resistance to tamoxifen. After a genome-wide profiling study in MBC patients, EZH2 showed to have higher expression levels in patients with resistance to first-line tamoxifen. EZH2 is a histone methyltransferase that mediates trimethylation of lysine residue 27 on histone 3 (H3K27me3) in order to control transcriptional processes of several target genes. This way, EZH2 is involved in the initiation and progression of several types of cancer and seems to be an important prognostic marker. In this chapter we have explored the predictive value of EZH2 using primary tumors of patients and cell line models. We demonstrated that MBC patients with higher expression levels of EZH2 in the primary tumor have a shorter progression-free survival (PFS) during treatment with first-line

tamoxifen. Downregulating the expression of EZH2 using siRNAs in a cell line model (MCF7) showed less tumor growth and an even stronger effect when ICI 164383, as surrogate for tamoxifen, was added. Moreover, we discovered that less expression of EZH2 in this cell line model was associated with an upregulation of the expression of ER which can explain the cumulative response when adding ICI.

In **chapter 3**, we took a closer look at the EZH2 pathway. We investigated the quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) data from primary tumors of MBC patients treated with first-line tamoxifen. Through pathway analysis, we checked which genes were associated with a shorter PFS and thus with resistance.
High levels of miR-26a turned out to be associated with a favorable outcome in this setting, which can be explained through the inhibiting effect of miR-26a on EZH2. By investigating significantly differentially expressed genes between tumors with high and low expression levels of miR-26a and EZH2, it turned out that the genes associated with the cell cycle, CCNE1 and CDC2, were the only overlapping genes. Higher expression levels of these genes were associated with an unfavorable outcome. This can be explained by the CDC2 mediated phosphorylation on EZH2 which has an activating effect. The best response to tamoxifen can be expected in patients with low expression levels of EZH2, CCNE1 and CDC2 and high expression levels of miR-26a in their primary tumors.

Next to the generated data on mRNA level, we investigated whether protein levels of EZH2 are also involved in the response to tamoxifen in **chapter 4**. We therefore used a tissue microarray (TMA) which consisted of the primary tumor specimens of 250 MBC patients treated with first-line tamoxifen. The expression of EZH2 in tumor cells was scored on quantity and intensity and associated with PFS. With this study it became clear that also protein levels of EZH2 can be used to predict the response to tamoxifen since tumors with the highest expression of EZH2, independent of intensity, turned out to have a worse response to tamoxifen.

In **chapter 5** we investigated whether EZH2 is also involved in the response to AI. We used chromatin immunoprecipation (ChIP) and sequencing techniques to determine where ER binds to the DNA in tumors of patients treated with first-line AI. Furthermore, we determined in which areas of the genome the DNA is either accessible (H3K4me3) or not (H3K27me3) for ER in these tumors. The tumors showed unique binding patterns for ER, H3K27me3 and H3K4me3 that marginally overlapped. The clearest difference between DNA binding events was shown between ER and H3K27me3 when comparing good and poor outcome patients treated with first-line AI. Next, these differential enriched binding patterns were validated in an independent patient cohort and showed to be predictive for response to AI. These results suggest that the accessibility of the genome can determine the behavior of ER and that H3K27me3, as a sign of activity of EZH2, is predictive for a poor response to AI.

Finally, we used gene expression in CTCs to predict the response to AI in **chapter 6**. CTCs can be detected in the peripheral blood. It is thought that CTCs are a better representation of the metastases since differences between the primary tumor and metastases are increasingly recognized. These differences are caused by the course of time, genomic instability of a tumor and exposure to treatment.

In this study, we isolated CTCs of 45 MBC patients treated with first-line AI and molecularly characterized them using a previously selected 96-gene profile. Next, we investigated which genes were most differentially expressed between patients with a PFS shorter and longer than 9 months. From this selection an 8-gene profile was generated that happened to best identify patients with a good response to AI therapy. Although these results require validation, they can contribute to a more personalized treatment approach.

In the end, the main findings of this thesis are discussed and placed in a broader perspective in **chapter 7**. Both the underlying mechanisms of the findings as well as the limitations of the study are reviewed. The chapter concludes with suggestions for future research.

In conclusion, in this thesis we have demonstrated that a higher expression level of EZH2 in the primary tumor of MBC patients is associated with a worse response to tamoxifen on both mRNA and protein level. The same holds true for higher expression levels of CDC2 and CCNE1 and a lower expression level of miR-26a which are all factors involved in the EZH2 pathway. Downregulation of EZH2 seems to be a good therapeutic strategy because it increases the sensitivity to tamoxifen which might be due to a higher expression of ER.
The activity of EZH2, measured through H3K27me3 binding events, has shown to be predictive for a shorter PFS during treatment with first-line AI. Finally, molecular characterization of CTCs can be used to identify patients that respond to AI therapy.

It might be possible that due to the findings in this thesis EZH2 and gene expression analyses of CTCs will get involved in the determination of the response profile of a patient to come to a personalized and more targeted treatment decision.

SAMENVATTING

De afgelopen jaren is kanker de meest voorkomende doodsoorzaak in Nederland geworden. Borstkanker is bij vrouwen de meest voorkomende kankersoort. In de meeste gevallen betreft dit een laag stadium borstkanker met goede prognose. Deze prognose is afhankelijk van het ziektestadium bij diagnose en omvat de grootte van de tumor, wel/geen aangedane lymfeklieren en de aan- of afwezigheid van metastasen op afstand. Ook de moleculaire classificatie is een belangrijke prognostische en predicitieve factor waarvan de bekendste factoren de oestrogeen receptor (ER), de progesteron receptor (PR) en HER2-neu zijn. De ontwikkeling van therapieën gericht op de ER heeft de prognose van borstkanker enorm verbeterd en wordt geschaard onder de endocriene therapie. Tamoxifen, een selectieve oestrogen receptor modulator, speelt al meer dan 30 jaar een hoofdrol in de behandeling van borstkanker. Het gaat de competitie aan met oestradiol om aan de ER te kunnen binden en onderdrukt de transcriptionele activiteit van ER door een conformatie verandering in de eiwitstructuur van de receptor te induceren.

Een andere groep medicamenten binnen de endocriene therapie wordt gevormd door de aromatase remmers (AI). Door het remmen van het aromatase enzym wordt er minder oestradiol gevormd en is hiermee minder hormoon beschikbaar voor de receptor en dientengevolge voor de tumor.

Endocriene therapie is ook in de gemetastaseerde setting een veelgebruikte vorm van therapie, met name vanwege het gunstige toxiciteitsprofiel. Hoewel veel patiënten goed reageren op deze vorm van behandeling is bekend dat circa 30% van de patiënten met gemetastaseerde borstkanker (MBC) reeds bij aanvang van de behandeling niet responderen vanwege (intrinsieke) resistentie. Tevens zal iedere behandelde patiënt uiteindelijk resistentie ontwikkelen. Hierdoor is MBC nog steeds een ongeneeslijke ziekte, ondanks de huidige kennis van de ziekte en de ontwikkeling van nieuwe therapieën. Het beter begrijpen van de mechanismen betrokken bij resistentie zal kunnen leiden tot een verandering in behandeling en hiermee tot uitstel van het optreden van resistentie. Met het genereren van biomarkers kan per patiënt een beter en gerichter geïndividualiseerd behandelplan opgesteld worden. Daarom, zoals beschreven in **hoofdstuk 1**, is het doel van dit proefschrift om (epigenetische) factoren betrokken bij endocriene therapie resistentie te ontdekken en te begrijpen. Om tot dit doel te komen is gebruik gemaakt van cellijnmodellen naast zowel primaire tumoren als circulerende tumorcellen (CTCs) afkomstig van patiënten.

Hoofdstuk 2 beschrijft de bevinding dat Enhancer of Zeste Homolog 2 (EZH2) betrokken is bij tamoxifen resistentie. EZH2 was uit een eerdere *genome-wide* studie naar voren gekomen als een gen dat hoger tot expressie komt in patiënten met MBC met resistentie tegen eerstelijns therapie met tamoxifen. EZH2 is een histon methyltransferase en in staat om van verschillende targetgenen histon 3 lysine residu 27 te trimethyleren (H3K27me3) en zo de expressie hiervan te veranderen. EZH2 is op deze manier betrokken bij het

ontstaan en de progressie van diverse tumorsoorten en lijkt hiermee een belangrijke prognostische marker te zijn. In dit hoofdstuk hebben we de predictieve waarde van EZH2 onderzocht waarbij we gebruik hebben gemaakt van tumormateriaal afkomstig van patiënten en cellijnmodellen. We hebben aangetoond dat de patiënten met MBC met een hoge expressie van EZH2 in de primaire tumor een kortere progressievrije overleving (PFS) hebben gedurende behandeling met tamoxifen. Bij het verminderen van de expressie van EZH2 met behulp van zogenoemde siRNA's in een cellijnmodel (MCF7) bleek dat de tumorcellen langzamer gingen groeien en dat dit versterkt werd wanneer ICI 164383, als surrogaat voor tamoxifen, werd toegevoegd. Tevens zagen we in dit cellijnmodel dat verminderde expressie van EZH2 was geassocieerd met een verhoging van de expressie van ER, wat een goede verklaring kan zijn voor de versterkte respons op het toevoegen van ICI.

In **hoofdstuk 3** zijn we dieper ingegaan op de signaaltransductie route waar EZH2 op ingrijpt. We hebben gekeken naar de Real-Time Polymerase Chain Reaction (qRT-PCR) data verkregen vanuit de primaire tumoren van patiënten met MBC en behandeld met eerstelijns tamoxifen. Door middel van pathway analyse kon gekeken worden welke genen corresponderen met een korte PFS en daarmee met resistentie. Hoge gehaltes van miR-26a bleken gunstig te zijn waarbij dit te verklaren valt door de remmende werking van miR-26a op EZH2. Door te onderzoeken welke genen significant anders tot expressie kwamen tussen tumoren met respectievelijk hoge en lage expressie van miR-26a en EZH2, werd duidelijk dat de celcyclus geassocieerde genen CCNE1 en CDC2 de enige overlappende genen waren. Verhoogde expressieniveaus van deze genen bleken gerelateerd aan een slechtere uitkomst. De verklaring kan liggen in de fosforylerende, en daarmee activerende, werking van CDC2 op EZH2. De beste respons op tamoxifen is derhalve te verwachten bij patiënten met lage expressieniveaus van EZH2, CCNE1 en CDC2 en hoge expressie van miR-26a in hun primaire tumoren.

Naast de gegenereerde data op mRNA niveau, hebben we in **hoofdstuk 4** gekeken of EZH2 ook op eiwitniveau betrokken is bij de respons op tamoxifen. Hiertoe hebben we gebruik gemaakt van een zogenoemde tissue microarray (TMA) welke bestond uit de primaire tumor samples van 250 MBC patiënten behandeld met eerstelijns tamoxifen. De expressie van EZH2 in de tumorcellen werd gescoord op hoeveelheid en intensiteit en gecorreleerd aan PFS. Vanuit deze studie is gebleken dat EZH2 ook op eiwitniveau gebruikt kan worden om de uitkomst op tamoxifen te voorspellen daar de tumoren met de meeste EZH2 expressie, onafhankelijk van de intensiteit, een slechtere respons op tamoxifen hebben.

In **hoofdstuk 5** hebben we gekeken of EZH2 ook een rol speelt in de respons op AI. We hebben chromatine immunoprecipitatie (ChIP) en sequencing technieken gebruikt om te bepalen op welke plekken in het genoom ER aan het DNA bindt in de primaire tumoren van patiënten met MBC die zijn behandeld met eerstelijns AI en die een goede of een slechte uitkomst hadden. Hiernaast hebben we bij deze tumoren gekeken in welke gebieden van het genoom het DNA wel (H3K4me3) of niet (H3K27me3) toegankelijk is

voor ER. De tumoren bleken unieke ER-, H3K27me3- en H3K4me3-bindingspatronen te bezitten met weinig overlap. Vooral het DNA-bindingspatroon van ER en het DNA-toegankelijkheids-profiel van H3K27me3 verschilde voor specifieke genen tussen patiënten met een goede of slechte respons op AI. De expressie van deze specifieke genen van het ER- en het H3K27me3-bindingspatroon bleken vervolgens in tumoren van een onafhankelijke groep patiënten voorspellend te zijn voor de respons op AI. Deze resultaten suggereren dat de toegankelijkheid van het genoom het gedrag van ER kan bepalen en dat H3K27me3, oftewel de activiteit van EZH2, voorspellend is voor een slechte respons op AI.

Tot slot hebben we in **hoofdstuk 6** gebruik gemaakt van genexpressieprofielen in CTCs om de respons op AI te voorspellen. CTCs kunnen worden gedetecteerd in het perifere bloed. De veronderstelling is dat CTCs een betere representatie zijn van gemetastaseerde ziekte, mede gezien het bekend is dat er grote verschillen kunnen bestaan tussen de primaire tumor en de metastasen. Dit komt onder meer door de tijd, de genomische instabiliteit van een tumor en de blootstelling aan behandeling.
We hebben in deze studie de CTCs van 45 MBC patiënten behandeld met eerstelijns AI geïsoleerd en gekarakteriseerd middels RT-PCR door gebruik te maken van een eerder opgesteld profiel bestaande uit 96 genen. Vervolgens hebben we gekeken welke genen het meest differentieel tot expressie kwamen tussen patiënten met een PFS korter dan wel langer dan 9 maanden. Hieruit is een 8-gen profiel gegenereerd dat vooral in staat bleek om de patiënten te identificeren die goed reageren op eerstelijns AI therapie. Hoewel de resultaten uit deze studie nog validatie behoeven, is het een stap in de richting van een biomarker die de gewenste individuele therapie kan voorspellen.

Tenslotte worden in **hoofdstuk 7** de belangrijkste bevindingen van dit proefschrift bediscussieerd en in een breder perspectief geplaatst. Zowel de mogelijke onderliggende mechanismen van de bevindingen als de beperkingen van de studies worden besproken. Het hoofdstuk sluit af met suggesties voor toekomstig onderzoek.

Concluderend, in dit proefschrift hebben we op zowel mRNA- als eiwitniveau aangetoond dat een hoge expressie van EZH2 in de primaire tumor van patiënten met MBC geassocieerd is met een slechte respons op tamoxifen. Hetzelfde geldt voor een hoge expressie van CDC2 en CCNE1 en een lage expressie van miR-26a waarbij deze factoren betrokken zijn bij EZH2 activiteit. Het verlagen van de EZH2 expressie lijkt een goede therapeutische strategie doordat de gevoeligheid voor tamoxifen hiermee toeneemt en dit lijkt te berusten op een verhoogde expressie van ER.
De activiteit van EZH2, gemeten middels de H3K27me3 binding, blijkt eveneens voorspellend voor een slechtere respons op AI. Tot slot lijken CTCs goed gebruikt te kunnen worden om, middels moleculaire karakterisatie hiervan, patiënten te identificeren die goed responderen op AI therapie.

Mogelijk is door de bevindingen van dit proefschrift een toekomstige rol voor EZH2 en CTC-analyses weggelegd in de bepaling van het responsprofiel van een patiënt om hiermee tot een geïndividualiseerd, en daarmee gerichter, behandelplan te kunnen komen.

APPENDIX I

Dankwoord

DANKWOORD

Daar ben ik dan, aangekomen bij het dankwoord. Het meest gelezen deel van dit proefschrift en des te spannender is het om dit stuk te schrijven. Het is alweer even geleden dat mijn promotietijd begon en de afgelopen jaren is er veel gebeurd. Ik wil beginnen met het bedanken van een ieder die op wat voor manier dan ook een rol in mijn leven heeft gespeeld. Een aantal personen wil ik hieronder graag bij naam noemen.

Allereerst en in het bijzonder mijn promotoren. Prof.dr. Berns, lieve Els, mijn wetenschappelijke moeder. Ik zou bijna willen zeggen dat dit proefschrift er niet was geweest als jij niet zo volhardend in mij was blijven geloven. Op het moment dat ik het nauwelijks meer zag zitten, heb je me weten op te peppen om door te gaan en dat ene stapje meer te zetten. Het afgelopen jaar hebben we er samen hard aan gewerkt en ik hoop dat je net als ik trots bent op het eindresultaat. Ik bewonder je manier van leven en je enthousiasme voor het onderzoek en onderwijs. Ik ben blij dat ik er een vleugje van heb meegekregen. Geniet van je welverdiende pensioen, het leven zit vol mooie dingen!

Prof.dr. Sleijfer, beste Stefan, mijn interesse in de oncologie is bij jou in de polikamer begonnen. Vele jaren later kan ik zeggen dat dit proefschrift er zonder jou niet was geweest. Ik was de afgelopen jaren erg enthousiast voor andere dingen en de angst om je teleur te stellen werd steeds groter waardoor ik menigmaal struisvogelpolitiek heb bedreven. Ik ben blij dat je toch altijd deed wat je moest doen in de rol van promotor en hoop dat je trots bent dat het nu eindelijk af is. Ik bewonder je tomeloze energie voor de kliniek en het onderzoek, je reactiesnelheid op mailtjes en verzoek tot nakijkwerk, je scherpte hierin en je gave om van ieder paper een juweeltje te maken door zinnen nét iets anders te formuleren zodat het een pakkend verhaal wordt. Dank voor al je inzet en begeleiding!

Prof.dr. Foekens, beste John, hoewel je nu in mijn grote commissie zit vind ik dat je een plekje bovenaan het dankwoord verdient. Je had aanvankelijk de rol van promotor en hoewel we in die hoedanigheid stiekem weinig met elkaar te maken hadden, heb ik je input altijd erg gewaardeerd. Je bent kritisch en scherp en weet als geen ander zelfs de verkeerd geplaatste punten en komma's uit een stuk te halen. Daarnaast ken ik niemand die zó goed op de hoogte is van de literatuur; zelfs mijn referentielijsten wist je te corrigeren.

Prof.dr. Touw, beste Ivo, dank voor je rol als secretaris van mijn kleine commissie en het lezen en beoordelen van mijn proefschrift. We hebben vaak vergaderd over het reilen en zeilen rondom promovendi en ik kijk terug op een tijd waarin we, met een blik op de huidige gang van zaken tijdens een promotietraject, een hoop positieve veranderingen voor elkaar hebben weten te krijgen. Derhalve vind ik het des te leuker dat we nu in een andere hoedanigheid tegenover elkaar staan.

Beste prof.dr. Van Kemenade en prof.dr. Linn, dank voor het plaatsnemen in de kleine commissie en de tijd en moeite die de beoordeling van het proefschrift van jullie heeft gevraagd.

Prof.dr. Van Laere, beste Steven, dank voor het naar Nederland afreizen om met mij in discussie te gaan over de bevindingen van dit proefschrift. Ik kijk nog steeds met veel plezier terug op de samenwerking en het daarbij behorende memorabele etentje in Antwerpen.

Beste dr. Stouthard, beste Jacqueline, ook u wil ik graag bedanken voor het plaatsnemen in mijn commissie. Ik zie het als een mooi begin van een samenwerking in het Antoni van Leeuwenhoek ziekenhuis; ik ben ervan overtuigd dat ik veel van u kan leren en kijk erg uit naar deze periode.

Prof.dr. Zwarthoff, dank voor uw bereidheid met mij van gedachten te wisselen over de inhoud van dit proefschrift.

Gedurende het onderzoek heb ik een hoop geleerd en ben ik uitstekend begeleid door alle medewerkers van het lab. Ik heb een erg leuke tijd gehad en wil dan ook iedereen hiervoor bedanken. Enkele personen in het bijzonder. Dr. M.P.H.M. Jansen, beste Maurice, ik heb de afgelopen jaren getraind op jouw voorletters en kan ze nu op ieder willekeurig moment uitspellen. Dank voor al je geduld en je goede begeleiding. Ik heb je harde werk altijd erg gewaardeerd. Ik vind het knap om te zien hoe je je werk met passie uitvoert en daarnaast een groot gezin draaiende weet te houden; dat zal, zeker gezien de gebeurtenissen, niet altijd makkelijk zijn.

Anieta, zonder jou waren vele CTCs niet geïsoleerd en vele samples niet door de PCR gegaan. Ik ken niemand die deze techniek zo goed beheerst als jij. Jouw berekeningen en normalisaties zijn me altijd een raadsel geweest. Jouw werkritme overigens ook ;). Dank voor je tomeloze inzet voor de CTC-studies!

Marcel Smid, dank voor al je analyses de afgelopen jaren! Vraag na vraag heb je altijd geduldig beantwoord, hoewel er vast een aantal minder intelligente tussen hebben gezeten. Dank ook voor je nuchtere houding, de appelrondes en de gezelligheid.

Joan, de spil van het lab. Ik heb vaak gedacht dat je niet met pensioen kan gaan en nu ik al 3 jaar weg ben van het lab, krijg ik die indruk nog steeds op de momenten dat ik er ben. Je houdt het lab in al zijn vezels draaiende, dank daarvoor!

Kirsten en Iris, de twee technici die mij de kneepjes van het vak hebben geleerd. Iris, de vele uren samen in de celkweek om de si-experimenten uit te voeren, ongeacht de dag van de week, vond ik erg leuk en leerzaam. Ik vond het jammer dat je het lab hebt (moeten) verlaten, maar volgens mij heb je genoeg andere werkzaamheden waarin je al je energie kwijt kan.

Kirsten, de meest secure persoon die ik ken! Vele experimenten waren vast mislukt als jij er niet was geweest. Wat heb je een oog voor detail en wat weet je goed hoe het allemaal werkt! Ook jou bewonder ik voor je combinatie werk-privé; het is niet altijd makkelijk geweest, ik hoop dat je je draai hebt gevonden.

Jean en Wendy, zonder jullie waren er een stuk minder data! Jean, je hebt een hoop experimenten gedaan en ik vond het altijd mooi om te zien hoe je ze allemaal wist te analyseren. Wendy, jij kan vast geen gel of Western Blot meer zien. Je hebt veel geoptimaliseerd en uitgevoerd, daar heeft iedereen vast nog profijt van. Leuk dat je nu een andere weg op bent gegaan, ik weet zeker dat die je heel goed ligt!

Jozien en Corinne, dank voor het samenwerken. Jullie zaten op andere projecten, maar de vele werkbesprekingen over mijn projecten zijn door jullie visie zeker beter geworden.

John Martens, qua werk hebben we niet veel met elkaar te maken gehad, maar jouw aanwezigheid tijdens de CTC-besprekingen vond ik altijd erg waardevol. Jouw intelligentie en brede kennis zijn een kostbaar goed. Knap hoe je bent uitgegroeid tot een ware werkgroepleider.

Mieke Timmermans, dank voor het mede-beoordelen van de gehele TMA. Ik ben trots op het paper dat daaruit voortgekomen is. Maxime, dank voor al je statistische analyses.

Arzu, dank voor jouw altijd motiverende woorden tijdens onze ontmoetingen op de gang. Ik heb er veel aan gehad! Fijn dat je een nieuwe uitdaging hebt gevonden die je goed bevalt.

Dr. Kraan, beste Jaco, Sjakie! Ik kan hier moeilijk gaan zeggen dat jij mijn favoriete roomie was, want stiekem hadden we allebei een ander. Maar toch, wat heb ik een leuke tijd met je gehad! Het was niet alleen gezellig op onze kamer (althans, dat vond ik), maar er was zeker ook ruimte om te ventileren. Soms had ik daar enige muzikale ondersteuning of spanddoek-uitspatting bij nodig zoals je vast hebt gemerkt. Dank voor al je medewerking, vooral in de laatste fase van mijn proefschrift!

Ook wil ik alle co-auteurs bedanken voor hun bijdrage aan de artikelen waaruit dit proefschrift bestaat. Mijn specifieke dank gaat uit naar alle oncologen uit het Erasmus MC die hebben meegewerkt aan de inclusie in de CTC 09-405 studie. Tevens wil ik dr. Paul Hamberg uit het Sint Franciscus Gasthuis en dr. Felix de Jongh uit het Ikazia Ziekenhuis bedanken voor hun tomeloze inzet bij de inclusie in deze studie en het kritisch beoordelen van het manuscript dat uiteindelijk heeft geleid tot het 8-gen paper. Dank ook aan alle research verpleegkundigen uit de diverse centra. Zonder jullie inzet is het niet mogelijk om alle logistiek zo strak georganiseerd te krijgen.

Niet op de minste plaats behoort hier ook mijn dank aan alle patiënten voor hun belangeloze bijdrage aan het wetenschappelijk onderzoek en het hierdoor van zeer grote betekenis zijn voor de toekomst van de oncologie met haar toekomstige patiënten. De goede samenwerking tussen de afdeling Interne Oncologie en Pathologie (Carolien van Deurzen) heeft geresulteerd in een aantal mooie papers en dank ik hiervoor.
Ook de samenwerking met het *Center for Oncological Research* van het Sint-Augustinus Ziekenhuis/Universiteit van Antwerpen wil ik hartelijk danken. Prof.dr. Steven van Laere en prof.dr. Luc Dirix, dank voor al jullie input tijdens de inter-lab meetings. Dieter, dank voor al jouw werk voor de CTC 09-405 studie.

Promeras, wat een mooie tijd hebben we gehad! Martijn, Jacqueline en Anne; ik zat het grootste gedeelte met jullie in het bestuur en vond het niet alleen gezellig, maar ook erg leerzaam. Ik kijk met veel plezier terug op alle activiteiten. Alle leden van de PhD-committee, dank voor alle leerzame momenten; ik heb mijn liefde voor bestuurlijke activiteiten bij jullie ontwikkeld.

Alle mede-promovendi door de jaren heen: Bianca, Wendy, Arjen, Nick, Marjolein, Inge, Lisanne en Lindsey en van de 'andere groep' Sander, Jacqueline, Anne-Joy en Annemieke. Bianca, dank voor al je pioneerswerk op het gebied van de CTCs. Mooi om te zien dat je zowel de kliniek als het onderzoek combineert alsmede werk en privé. Ik heb stiekem meer aan je gehad dan je weet; jouw proefschrift is voor mij een voorbeeld en drijfveer geweest!
Nick, een vreemde eend in de bijt na Stefan's angels maar je hebt je kranig geweerd. Veel succes in de kliniek!
Inge, leuk dat je eerst mijn Amphia-collega was en nu mijn onderzoekscollega; dat belooft wat voor de toekomst!
Sander, dank voor je gezelligheid en jouw passie voor wielrennen. Die is zeker op mij overgeslagen nadat ik aanvankelijk noodgedwongen de Stelvio op moest toen mijn naam ineens op de reclameposter verscheen als onderdeel van het fietsteam zonder dat ik een racefiets had, laat staan er ooit op één had gezeten. Ik kijk met veel plezier terug op onze tijd in Bormio!
Mooi om te zien dat de AIO-groep inmiddels flink is uitgebreid; dit levert vast mooie resultaten op. Ik wens iedereen succes!

Wendy, dr. W., mijn favoriete roomie en partner in crime! Jammer dat de écht goede grappen werden uitgevoerd toen ik al in Breda zat, maar onze ondernemingen om Jaco te laten genieten van onze aanwezigheid (met spandoek in het zwembad, op zoek in Bergambacht; dit laatste heeft overigens jammerlijk gefaald) vond ik geweldig. Fijn dat je een luisterend oor had als ik het gevoel kreeg in de huid van Ariel te moeten kruipen, maar ook op serieuze momenten om onze perikelen, op welk gebied dan ook, te bespreken. Ik kijk met veel plezier terug op onze tijd in het JNI, in onze villa in Amsterdam en in onze wolkenkrabber in Chicago (mét Walmart in de kelder die na ons bezoek, hoewel, vooral na jouw bezoek, 30 lipgloss minder rijk was). Hopelijk creëren we nog meer mooie momenten in de toekomst! Dank dat je op deze bijzondere dag mijn paranimf wilt zijn!

Lieve bazen uit het Amphia ziekenhuis, bedankt voor jullie begeleiding, geduld en kennisoverdracht. Daarnaast dank voor alle gezelligheid, onder meer tijdens de borrels en skiweekenden. Tevens wil ik jullie allen danken voor jullie tomeloze vertrouwen in een succesvolle afloop van mijn promotietraject. Ik heb me altijd erg thuis gevoeld in het Amphia, heb enorm veel geleerd en vind het dan ook jammer dat mijn tijd er bijna op zit. De sfeer in het ziekenhuis en in de maatschap heb ik altijd als bijzonder goed en vertrouwd ervaren. Jullie allen vormen een voorbeeld voor hoe ik het later graag zou willen hebben en doen. In het bijzonder wil ik enkele mensen noemen.
Allereerst dr. Van Esser, beste Joost, wat ben ik blij met een opleider als jij! Je bent niet alleen een goede dokter met veel kennis van zaken, maar ook een goede opleider. Je creëert een prettige werksfeer voor alle assistenten. Ik hoop, mocht ik ooit opleider worden, dat ik op je zal lijken. Dank ook voor de prettige samenwerking in mijn eerste jaren om de zaken zo goed mogelijk te organiseren binnen de assistentengroep. Als Toos heb ik dit samen met Bep altijd met veel plezier gedaan. Ook dank voor je optreden als referent tijdens mijn sollicitatie!
Dr. Van Guldener, beste Coen, in mijn eerste jaar was jij nog de opleider en heb ik je vanaf het begin bewonderd. Jouw kennis is eindeloos en jouw onderwijsvaardigheden zijn briljant. Ik ben blij en voel me vereerd dat ik onder jouw supervisie in het Amphia ziekenhuis heb mogen werken.
Drs. Ennecker, beste Simone, dank voor je vertrouwen en stimulatie om mijn proefschrift af te ronden.

Lieve mede arts-assistenten uit het Amphia, dank voor jullie collegialiteit en gezelligheid! Ook alle collega's van mijn stages bij de Longgeneeskunde, Cardiologie en Intensive Care wil ik hartelijk danken voor de begeleiding en leuke en leerzame tijd.
Jurjen, bijna dr. Versluis, labrador, lieve Bep. We zijn samen begonnen aan onze tijd in het Amphia ziekenhuis en vanaf het begin onafscheidelijk geweest. Hoewel we compleet andere personen zijn, klikte het vanaf het eerste moment. Ik bewonder je discipline, intelligentie en relaxte houding. Dank voor de samenwerking en je steun, dank voor alles!
Lieve mede-Alpha, lieve Sophie, wat ben ik blij met een collega als jij. We delen niet alleen onze liefde voor de oncologie, maar ook voor kleding, lekker eten, slechte grappen en gezelligheid. Ik vind het erg bijzonder dat je naast en achter me staat op deze dag. Dank voor alles! We gaan er een mooie tijd van maken in Amsterdam.
Lieve Danick, ik kijk terug op een fijne tijd in de flexkamer tijdens mijn poli- en consultenstage. Je verlicht de dagen met jouw eeuwige enthousiasme en energie. Ik ken ook niemand die zo attent is als jij! Jurjen, Sophie en Danick, hopelijk volgen er nog vele Alpha-dates!
Diane, wat heb ik een geluk gehad dat ik op mijn eerste dag door jou werd ingewerkt. Ik weet dat jij er anders over denkt, maar voor mijn gevoel is in dat hokje op afdeling 27 (de assistentenkamer) de basis gelegd voor een mooie vriendschap. Ik vind onze band erg bijzonder en koester deze. Je bent een lieve vriendin!
Eva, wat ben jij een kanjer! Ik ben trots op alles wat je hebt bereikt en bewonder je

drive. Daarnaast geniet ik van onze oeverloze gesprekken en slechte grappen. Dank voor je gezelligheid maar bovenal dank voor je vriendschap!
Lieve Sophia, hoewel we niet lang collega's zijn geweest klikte het meteen tussen ons. Onze koffie- en eetdates zijn momenten waar ik altijd naar uitkijk. Onze vakantie naar Maleisië is wat mij betreft een absoluut hoogtepunt van onze vriendschap. Op naar meer fijne momenten!
Lieve Carlijn, jouw aanwezigheid in mijn leven geeft het leven kleur! Ik kan met je lachen, huilen en carnaval met je vieren: de perfecte basis om onze vriendschap nog lang te laten bestaan. Je bent altijd welkom om te blijven logeren, ook als ik niet meer in Rotterdam woon!

Lief (ex-)hockeyteam, 22 jaar van mijn leven heb ik met jullie lief en leed gedeeld. Jullie waren van alles het eerst op de hoogte en samen hebben we ups en downs beleefd. Ik vind het fantastisch dat we elkaar, ook zonder trainingen en wedstrijden, niet uit het oog verloren zijn. In het bijzonder wil ik hier enkele teamgenoten/vriendinnen noemen.
Lieve Andrea, onze vriendschap begon eigenlijk al op de middelbare school maar kwam pas echt van de grond toen we bij elkaar in het team kwamen. We hebben er inmiddels vele mooie avonden en wintersportvakanties op zitten en ik weet zeker dat we dit gaan voortzetten. Ik ben blij dat je je liefdesgeluk hebt hervonden!
Lieve Charlène, ik word altijd blij van jou! Je bent lief, grappig en behulpzaam. Ik moet altijd lachen als ik terugdenk aan de, soms wat knullige, dingen die we hebben meegemaakt. Dank voor alle ritjes Rotterdam-Capelle waarin we elkaar op de hoogte stelden van het wel en wee in onze levens. Ik vind het erg bijzonder dat ook jouw moeder een belangrijke rol mag spelen op deze memorabele dag.
Lieve Bianca, je bent sterk in alles wat je doet en beleeft. Hoewel niet altijd alles even makkelijk gaat, weet je er voor een ander te zijn en ook je eigen dromen na te leven. Bijzonder! Geniet van het leven!
Lieve Wendelijn, ik moet altijd lachen met (en ja, ook om) jou! In jou heb ik iemand gevonden die nóg meer praat dan ik. Dank voor alle fijne momenten rondom de hockey, tijdens de wintersport en alle dagen en avonden dat we afspreken. Laat er nog vele volgen!
Lieve Marieke, ik bewonder je nuchtere houding en discipline in het leven. Je hebt het al ver geschopt, ik ben trots op je! Je komt uit een warm gezin waar ik ook altijd graag kom; dank aan jouw ouders voor hun eeuwige interesse in mijn leven en werkzaamheden.
Victor, ook jij verdient hier een plekje. Hoewel je altijd wat afstandelijk overkomt, merk ik jouw bezorgdheid en de daaruit voortvloeiende zorgzaamheid en waardeer ik deze.
Lieve Nathalie, dank voor alle momenten dat ik weer eens dreigde te laat te komen voor een training en jij nooit te beroerd was om door de drukte van de stad mij op te halen. Dank ook voor alle lekkernijen; ik blijf erbij, je zou er iets mee moeten doen!
Lieve Hilde, wat een bewonderingswaardig doorzettingsvermogen heb je! Leuk dat je je levensgeluk hebt gevonden; hopelijk gaat je gezondheid ook meewerken.
Lieve Chantal, hoewel je nooit bij mij in het team hebt gezeten, behoor je hier wel toe. Als mijn nicht ervaar ik een speciale band met jou en koester ik deze. Fijn dat we elkaar, zonder elkaar vaak te zien, op deze manier kennen!

Lieve Harvard homies, hoewel Boston inmiddels al ver achter ons ligt, werd hier de basis gelegd voor een vriendschap waarin het niet nodig is om elkaar vaak te zien, maar op de momenten dat het gebeurt, dit altijd een feest is. Lieve Denise, wat heb ik enorm veel respect voor jou! Jouw manier van leven waarin je niet bang bent om je kwetsbaar op te stellen, hard te werken, mooie reizen te maken en ultiem te genieten van het leven, vind ik mooi. Ik heb vertrouwen in jouw toekomst! Lot, onvoorstelbaar hoe jij je opleiding, promotie, verhuizing, trouwen, en het moederschap allemaal combineert of in elkaar laat overvloeien. De bezoekjes aan jou en Stijn voelen voor mij altijd als een kleine vakantie. Succes met de laatste loodjes van jouw proefschrift!

Lieve Kalynda, Gytha en Vicky, onze ontmoeting in Suriname was er één uit duizenden. Inmiddels 5 jaar geleden maar we zijn elkaar nadien nooit uit het oog verloren. Kalynda, dank dat je nooit schroomde om langs Rotterdam te rijden voor een etentje of borrel. In de toekomst kom ik vaker jouw kant op. Succes met al jouw plannen!
Gytha, jij weet hoe je van het leven moet genieten! Blijf dit doen; ik geniet graag mee. Leuk dat we binnenkort eindelijk bij elkaar in de buurt wonen, ik kijk er naar uit!
Vicky, hoewel we elkaar niet vaak zien, voelt onze vriendschap altijd vertrouwd. Dank daarvoor! Succes met alles, je komt er wel!

Lieve Eefje, onze vriendschap gaat al zo ver terug. Als Knabbel en Babbel hebben we de basisschool doorlopen en volgens mij voldoen we nog steeds aan die beschrijving. We zien elkaar niet vaak maar weten beiden dat dit niet nodig is. Fijn dat je (nog steeds) in mijn leven bent!

Dan mijn lieve Primaatjes! Zonder jullie in mijn leven was het niet zo leuk geweest! Iedereen is anders, iedereen betekent veel voor me en als groep zijn we ijzersterk. Dank jullie wel voor onze bijzondere vriendschap! Ik geniet van al onze momenten samen: etentjes, thee en/of wijn en/of gin tonics drinken, alle verjaardagen en alle mooie momenten tijdens onze tripjes; van vakanties naar de meest foute oorden tot kamperen in de Schaapskooi, Ermelo of onze verblijven in een luxe villa op Curaçao of Ibiza. Lieve Marcella, we go way back. Inmiddels kennen we elkaar 18 jaar en is het allemaal begonnen op brugklaskamp. Met een soort seintaal hebben we elkaar leren kennen en zijn we nooit meer uit elkaar gegaan. Je hebt een hoop hindernissen genomen en ik vind het knap om te zien hoe je je leven inmiddels hebt georganiseerd. Succes met je nieuwe opleiding!
Lieve Lianne, we go even more back! Geen idee hoe lang we elkaar precies kennen, maar de knutselclub was onze basis. Inmiddels knutselen we wat minder, maar dat mag de pret niet drukken. Wat hebben wij mooie en bizarre dingen beleefd! Op naar nog veel meer! Ik ben trots op jou als persoon; je doorstaat alle tegenslagen als geen ander en bent ondertussen huisarts geworden.
Lieve Irene, ook wij zijn van origine van de knutselclub. Jouw nuchterheid en stabiliteit waardeer ik zeer en ik vind het fijn om te merken dat het niet uitmaakt waar we wonen of hoe vaak we elkaar zien, de basis is daar. Ik ben trots op je!

Lieve Kelly, dank je wel voor je vriendschap voor al zo'n lange tijd. Ik bewonder je relaxte houding in het leven en je inzet bij alles wat je doet. Je was de eerste van ons die écht carrière maakte en je doet het fantastisch! Lieve Nadine, de absolute sfeermaker van de groep. Met jouw aanstekelijke lach en je hilarische verhalen weet je me altijd vrolijk te maken (of te houden). Ik bewonder hoe je je levenspad bewandelt en waardeer je creatieve geest.
Lieve Samantha, jouw cynische kijk op vele dingen in het leven doet me altijd realiseren waar het écht om gaat in het leven. Dank voor je luisterend oor op de momenten dat ik het nodig heb. Ik ben trots op de stappen die je hebt gezet en nog gaat zetten; geniet van het leven!
Lieve Anne, als mede-promovendus heb ik je discipline altijd bewonderd (en benijd). Hetzelfde doe ik nu je in opleiding bent tot neurochirurg. Ik weet zeker dat jouw promotie er ook gaat komen!

Lieve familie Brugman, dank voor jullie interesse in mijn leven en werkzaamheden. Dank ook voor alle leuke momenten samen, ik hoop dat er nog vele mogen volgen.
Lieve familie Reijm, het is fijn om uit zo'n grote familie te komen! Dank voor jullie interesse en gezelligheid. Zonder jullie aanwezigheid zijn de feestjes niet compleet. Mooi om te zien hoe de generaties zich uitbreiden met aan het hoofd oma Reijm. Oma, ik hoop dat u nog vele jaren de spil van de familie mag vormen!

Lieve familie van Roland, dank dat jullie mij zo snel hebben opgenomen in jullie gezin. Dank voor jullie interesse, waardering en warmte.

Lieve Rogier, dank voor het zijn van mijn grote broer. Je bent er altijd voor me en ik vind het fijn dat ik altijd bij je aan kan kloppen. Lieve Elena, dank voor het levensgeluk van mijn broer. Jouw zorgzaamheid is buitengewoon en ik bewonder hoe je het gezin met Fjodor en Jeanne draaiende houdt.
Lieve Agnes, jij bent mijn grote zus. Hoewel je dit misschien niet altijd zo voelt, is het toch echt zo. Ik ben trots op de stappen die je hebt gezet in je carrière en privé. Lieve Dennis, fijn om jou in de familie te hebben! Jij bent de rust zelve en ik waardeer je humor. Als trotse tante van Fenne kijk ik uit naar frequentere bezoeken in de toekomst.

Lieve pap en mam, dit boekje draag ik aan jullie op. Het is voor mij het symbool dat ik van jullie heb geleerd af te maken waar ik aan ben begonnen. Dank voor jullie onvoorwaardelijke steun en liefde. Dank ook voor jullie luisterend oor, oplossingen en geregel wanneer ik dit nodig heb. Jullie staan altijd voor me klaar. Kortom, dank jullie wel voor alles wat jullie me hebben (mee)gegeven.

Lieve Roland, ik kan niet beschrijven hoeveel je voor me betekent. Dank voor wie je bent, mijn liefde voor jou is intens. Jij bent mijn kompas, zowel op reis als in het leven. Jouw energie slaat op mij over en samen kunnen we de hele wereld aan. Je haalt het beste in mij naar boven, helpt me relativeren en te genieten van het leven. Dank voor je kracht, je steun en je liefde. Ik kijk uit naar onze toekomst samen, ik houd van je!

APPENDIX II

List of publications

van der Willik KD, Timmermans MM, van Deurzen CH, Look MP, **Reijm EA**, van Zundert WJ, Foekens R, Trapman-Jansen AM, den Bakker MA, Westenend PJ, Martens JW, Berns EM, Jansen MP. SIAH2 protein expression in breast cancer is inversely related with ER status and outcome to tamoxifen therapy. Am J Cancer Res. 2016 Jan 15;6(2):270-84. eCollection 2016.

Reijm EA, Sieuwerts AM, Smid M, Vries JB, Mostert B, Onstenk W, Peeters D, Dirix LY, Seynaeve CM, Jager A, de Jongh FE, Hamberg P, van Galen A, Kraan J, Jansen MP, Gratama JW, Foekens JA, Martens JW, Berns EM, Sleijfer S. An 8-gene mRNA expression profile in circulating tumor cells predicts response to aromatase inhibitors in metastatic breast cancer patients. BMC Cancer. 2016 Feb 18;16:123

Onstenk W, Sieuwerts AM, Weekhout M, Mostert B, **Reijm EA**, van Deurzen CH, Bolt-de Vries JB, Peeters DJ, Hamberg P, Seynaeve C, Jager A, de Jongh FE, Smid M, Dirix LY, Kehrer DF, van Galen A, Ramirez-Moreno R, Kraan J, Van M, Gratama JW, Martens JW, Foekens JA, Sleijfer S. Gene expression profiles of circulating tumor cells versus primary tumors in metastatic breast cancer. Cancer Lett. 2015 Jun 28;362(1):36-44.

Reijm EA, Timmermans AM, Look MP, Meijer-van Gelder ME, Stobbe CK, van Deurzen CH, Martens JW, Sleijfer S, Foekens JA, Berns PM, Jansen MP. High protein expression of EZH2 is related to unfavorable outcome to tamoxifen in metastatic breast cancer. Ann Oncol. 2014 Nov;25(11):2185-90

Jansen MP, Knijnenburg T, **Reijm EA**, Simon I, Kerkhoven R, Droog M, Velds A, van Laere S, Dirix L, Alexi X, Foekens JA, Wessels L, Linn SC, Berns EM, Zwart W. Hallmarks of aromatase inhibitor drug resistance revealed by epigenetic profiling in breast cancer. Cancer Res 2013 Nov; 73(22):6632-41

Maurice PHM Jansen, **Esther A Reijm**, Anieta M Sieuwerts, Kirsten Ruigrok-Ritstier, Maxime P Look, F. Germán Rodríguez-González, Anouk AJ Heine, John W Martens, Stefan Sleijfer, John A Foekens, Els MJJ Berns. High miR-26a and low CDC2 levels associate with decreased EZH2 expression and with favorable outcome on tamoxifen in metastatic breast cancer. Breast Cancer Res Treat 2012 Jun;133(3):937-47.

Reijm EA, Jansen MP, Ruigrok-Ritstier K, van Staveren IL, Look MP, van Gelder ME, Sieuwerts AM, Sleijfer S, Foekens JA, Berns EM. Decreased expression of EZH2 is associated with upregulation of ER and favorable outcome to tamoxifen in advanced breast cancer. Breast Cancer Res Treat 2011 Jan;125(2):387-94.

APPENDIX III

About the Author

ABOUT THE AUTHOR

Esther Anneke Reijm was born on May 20th 1986 in Hendrik-Ido Ambacht, the Netherlands. She grew up in Capelle aan den IJssel as the youngest of three children.
In 2004, she graduated from secondary school (Gymnasium) at the Comenius College in Capelle aan den IJssel. In the same year, she started her medical education at the Erasmus University of Rotterdam. During her second year she was selected to participate in the Master of Science program in Clinical Research at the Netherlands Institute for Health Sciences in Rotterdam. During this program she attended the summer school of the Harvard School of Public Health (HSPH), Boston, MA, United States of America. During 2008-2009, as part of both Medicine and the Master of Science program, she performed a research project at the department of Medical Oncology (*head*: Prof.dr. S. Sleijfer) on endocrine therapy resistance in breast cancer, under the supervision of prof. dr. J.A. Foekens and prof.dr. P.M.J.J. Berns.
Esther started the PhD project presented in this dissertation in 2009 but interrupted the project after a few months to finish her medical school. In the last year, as part of the clinical internships, she did an internship Emergency Medicine at the Academic Hospital in Paramaribo, Suriname. In 2009, she obtained the Master of Science degree in Clinical Research, and in 2011, she obtained her Medical degree (*cum laude*).
She continued the PhD traject fulltime during 2011 – 2013. During this time she was the treasurer of Promeras, the representing body of all PhD students at Erasmus Medical Center (MC). Besides, she was a member of the PhD committee of the Erasmus MC, the PhD committee of the Erasmus Postgraduate School of Molecular Medicine and took part in the workgroup focusing on internationalization. Next, she gave lectures in medicine and tutored first-year medical students.
In January 2014, she started working as a resident in Internal Medicine at the Amphia Hospital in Breda (*head:* Dr. C. van Guldener/Dr. J.W.J. van Esser) as part of her specialist training at the Erasmus Medical Center (*head:* Prof.dr. J.L.C.M. van Saase/Dr. S.C.E. Klein Nagelvoort). During her residency, she took responsibility for the working schedule of 25 – 30 residents, organized the yearly skiing trips and the resident weekends. Besides, she took part in several committees in order to come to a better hospital organization. In 2015, she got selected to participate in the winter school of the European School of Internal Medicine (ESIM) in Riga, Latvia.
During her working activities, she has always been an active member of the hockeyclub being the captain of the team and member of several committees. She also participated as a cyclist during the Stelvio for Life in 2013 in order to raise money for the Center for Personalized Cancer Treatment.
In 2017, she will temporarily interrupt her specialist training to travel in South America. In May 2017, she will resume this training at the Erasmus MC (*head:* Dr. S.C.E. Klein Nagelvoort), and in May 2018 she will start her training in Oncology at the Netherlands Cancer Institute – Antoni van Leeuwenhoek Hospital (*head:* dr. J.M.L. Stouthard).

APPENDIX IV

PhD portfolio

PHD PORTFOLIO

A summary of PhD training and teaching activities

Name PhD student:	Esther Anneke Reijm
Erasmus MC department:	Medical Oncology
Research school:	Postgraduate school Molecular Medicine
PhD period:	March 2009-December 2013
Promoters:	Prof.dr. S. Sleijfer and Prof.dr. P.M.J.J. Berns

1. **PhD training**	**Year**	**Workload (ECTS)**
General academic skills		
- Photoshop and Illustrator CS5 workshop	2012	0.3
- InDesign CS5	2012	0.2
- Research management	2012	1.0
- Workshop *Successful Grant Writing*	2012	0.3
- Workshop *Tutoring; how to handle a group*	2012	0.2
Research skills		
- Basic course regulations and organization for Clinical Researchers (Basiscursus Regelgeving en Organisatie Voor Klinisch onderzoekers [BROK[)	2012	1.5
In-depth courses		
- SNPs and Human Diseases	2008	2.0
- Basic and Translational Oncology	2008	1.8
- Molecular Medicine	2012	1.9
- Molecular Diagnostics VII	2012	1.0
Presentations		
- Poster and oral presentation at the FIGON Dutch Medicine Days	2008	1.3
- Annual oral presentation at the JNI Scientific Lab meetings	2009, 2013	1.0
- Poster presentation at the annual Molecular Medicine Day in Rotterdam	2009, 2012	0.6
- Poster and oral presentation at the Annual Meeting on Molecular Markers in Cancer	2009	1.3
- Poster presentation at the Annual San Antonio Breast Cancer Symposium	2009, 2012	0.6

-	Oral presentation at the annual scientific meeting of the Department of Medical Oncology	2009, 2012	1.0
-	Oral presentation at MTI	2012	0.3
-	Oral presentation at Daniel den Hoed Day	2012	0.3
-	Poster presentation at American Society of Clinical Oncology	2013	0.3

International conferences

-	Annual Meeting on Molecular Markers in Cancer in Brussels, Belgium	2009	1.0
-	Annual San Antonio Breast Cancer Symposium in San Antonio, TX, USA	2009, 2012	3.0
-	The European Cancer Congress, Amsterdam, The Netherlands	2013	1.0
-	American Society of Clinical Oncology in Chicago, Illinois, USA	2013	1.0

Seminars and workshops

-	Monthly JNI Oncology Lectures	2009, 2011-2013	0.9
-	Annual Molecular Medicine Day in Rotterdam	2009, 2012-2013	0.9
-	Annual Cancer Genomics Centre Scientific Meeting	2012	0.3
-	OMBO (Onderwijs Multidisciplinaire Behandeling In de Oncologie)	2012-2013	1.0
-	Borstkanker Behandeling Beter	2012	0.2

2. **Teaching activities**

-	Junior Med School	2008-2009, 2012	0.6
-	Supervision internship of HLO student	2012	2.0
-	Tutoring first-year medical students	2012	1.0
-	Tutoring first-year medical students in their acquaintance with the medical profession	2012	0.5
-	Lectures in medicine (minor Oncology)	2012	0.2
-	Lectures in medicine (second-year Keuze-onderwijs)	2013	0.2
-	Supervision internship Master student	2013	2.0

3. Other

Committees

-	Member of PhD Committee of the Erasmus Postgraduate School Molecular Medicine	2012-2013	1.0
-	Treasurer of Promeras, Erasmus MC, Rotterdam	2009-2013	5.0
-	Member of PhD Committee of the Erasmus MC, Rotterdam	2009-2013	1.4