ASPECTS OF GENETIC AND CLINICAL HETEROGENEITY IN BREAST CANCER.

ASPECTEN VAN GENETISCHE EN KLINISCHE HETEROGENITEIT VAN BORSTKANKER.

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR

AAN DE ERASMUS UNIVERSITEIT ROTTERDAM

OP GEZAG VAN DE RECTOR MAGNIFICUS

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EN VOLGENS BESLUIT VAN HET COLLEGE VOOR PROMOTIES

DE OPENBARE VERDEDIGING ZAL PLAATSVINDEN OP

14 DECEMBER 1994 OM 11.45 UUR.

DOOR

ATTE HENK BOOTSMA

GEBOREN TE RIJSWIJK

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Printed by: Haveka B.V., Alblasserdam, The Netherlands.

This thesis was supported by a gift from the Sandoz Research Foundation.

Voor Mariëtta en Nienke

Voor mijn ouders

CONTENTS

ABBREVIATIONS

APC	gene involved in familial adenomatous poliposis coli
AT	ataxia-telangiectasia
BRCA1	breast cancer 1 gene
cAMP	cyclic adenosine monophosphate
DCC	"Deleted in Colorectal Carcinoma" gene
DM	double minute
DNA	desoxyribonucleic acid
EGF	epidermal growth factor
ER	estrogen receptor
ERHSV1	enhanced reactivation of herpes simplex virus type 1
GH	growth hormone gene
HSR	homogeneously staining region
HSV-1	herpes simplex virus type 1
IGF-1	insuline-like growth factor-1
kb	kilobase
LOH	loss of heterozygosity
ß-MHC	ß-myosin heavy chain gene
MPO	myeloperoxidase gene
NER	nucleotide excision repair
NF1	neurofibromatosis type 1
NSE	neuron specific enolase
PR	progesteron receptor
RB	retinoblastoma tumour suppressor gene
SOS	international emergency signal
SSTR	somatostatin receptor
UV	ultra violet
VHL	Von Hippel Lindau gene
WT-1	Wilms tumour gene 1
XP	xeroderma pigmentosum

CHAPTER I: GENERAL INTRODUCTION

Breast cancer affects approximately 1 in every 12 women in Western countries. It is the leading cause of cancer death in women in these countries. Investigation of the mechanism of breast carcinogenesis is hampered by the heterogeneity of the disease that can be observed at the clinical, biological and genetic levels. Delineating this heterogeneity may give opportunities to gain more insight in the cellular mechanisms that lead to breast cancer. It may also be clinically helpful, by allowing diagnostic screening programmes to women at risk, and facilitating estimates of prognosis and the design of rational therapies adjusted to specific subgroups.

This thesis describes attempts to analyse various aspects of breast cancer heterogeneity from a phenotypic as well as from a genotypic point of view.

Genetic alterations are the fundamental changes that lead to cancer initiation and progression. Many genetic alterations have been observed in breast cancer and some of these changes could cause differences in histopathology of the tumour and in the clinical outcome of the disease. Our research therefore focussed on linking specific genetic events to subtypes of breast tumours or to an increased breast cancer risk.

CHAPTER II: INTRODUCTION

II.1: BREAST CANCER

Anatomy and physiology of the breast

The breast originates from the epidermal milk ridges on the ventral surface of the body. In the female it is composed of 5-9 separate glands that individually drain into the nipple. This glandular tissue is embedded in fibrous stroma that gives the breast its yielding consistency. Histologically the breast is composed of many branching ducts that on one side drain into the nipple and on the other side terminate in saccular glands: the duct lobules. The epithelial lining of the ducts consists of two cell types: the luminary secretory cells and the basal myoepithelial cells. The two can be differentiated by their specific keratin expression (keratin 7,8,18 and 5,14, respectively). The breast is a dynamic organ. Like the endometrium the breast epithelium starts to proliferate following the menstrual period under the influence of oestrogen. In the second half of the menstrual cycle stromal growth and oedema occur under the influence of progesterone. At the time of the menstrual period epithelial desquamation and atrophy of the stroma follow. Only during pregnancy the breast assumes its complete maturation with formation of true glands from the lobules[1,2].

Histology of breast cancer

Carcinomas derive from the epithelial lining of all parts of the excretory system of the breast, but are thought to occur principally at the transition zone from duct to lobule[3]. Carcinoma *in situ* is tumorous growth confined within the boundaries of preexisting tubules and/or lobules. The pathological characteristics and the clinical consequences of these lesions have not yet been completely determined and are still under investigation.

Carcinomas are routinely classified on morphological and topographic characteristics. (Table I)

TABLE I: Histological subtypes of breast cancer.

Non invasive carcinoma
intraductal carcinoma
lobular carcinoma, in situ
Invasive carcinoma
invasive ductal carcinoma ,not otherwise specified
medullary carcinoma
mucinous (colloid) carcinoma
papillary carcinoma
Invasive lobular carcinoma
tubular carcinoma
other rare types of carcinoma

However, the histopathology of a breast carcinoma is not the only informative parameter of the behaviour of the tumour and the prognosis of the patient. Patients are also stratified according to the extent of their disease (TNM classification) and to the differentiation grade of their tumour. In the TNM staging system T refers to tumour size, N to lymph node involvement and M to distant metastasis. Increasing stage number is correlated with a lower 5-year survival.

Tumours are also graded to their differentiation characteristics. Various grading systems are in use that are not completely satisfactory. At present many investigators put effort in the identification of better prognostic parameters with higher predictive value on tumour behaviour and disease outcome. Despite this effort, tumour size and lymph node involvement still are the most useful predictors of the patient's prognosis.

Incidence of breast cancer

Breast cancer is the leading cause of cancer deaths in females in Western countries. In the Netherlands over 7000 new cases are diagnosed each year and approximately 3000 women die of breast cancer annually[4,5]. The incidence in an unscreened population in the Southeastern Netherlands has doubled from 1960-1990[6]. This can in part be explained by a rise of the mean age of the population and to earlier diagnosis as a result of increased patient awareness and better screening programs. However, other as yet unidentified factors must also be involved.

Breast cancer incidence rises with age of the patient. Hardly any cases are seen under 25 years of age, after which the incidence rises 0.2% per woman-year until age 45 when a plateau is observed until age 55. From age 55 to 70 a second rise is observed from 0.2% to 0.3% of woman-years. The life time risk is around 8%. Interestingly the second rise in incidence is caused by an increase in the number of lobular and tubular carcinomas that is not seen in countries with a lower breast cancer incidence[7].

Risk factors for breast cancer

Both environmental as well as genetic factors apparently influence breast cancer risk:

1. Variation in incidence between countries has been observed. Northern and Western European and North American countries exhibit a much higher incidence than countries in Africa, Asia and Latin-America. This points to an effect of dietary habits and/or ethnic differences. The first possibility is supported by the observation that migrants take over the pattern of incidence of their new homeland.
2. The chances of getting breast cancer increase with age, reflecting longer exposure to carcinogenic stimuli.
3. Breast cancer risk increases with the duration of the exposure of breast

tissue, especially not fully differentiated breast tissue, to sex hormones: Late menarche, early full-term pregnancy and early menopause reduce breast cancer risk. Overweight increases the risk mainly in postmenopausal women in whom elevated levels of oestrogen are produced in adipose tissue.

4. A previous malignant breast tumour increases the risk for a second primary breast tumour five times.

5.Family history of breast cancer and the occurrence of this disease increases breast cancer risk. The relative risk for a daughter of a breast cancer patient being approximately three times and for a sister of a patient nine times the standard risk! The life time risk of getting breast cancer being 80% in those cases. This strongly supports the role of genetic factors in the etiology of breast cancer.

II.2: SOMATOSTATIN RECEPTOR-POSITIVE BREAST CANCER.

The experimental work presented in this thesis aims to investigate some aspect of the mechanisms involved in carcinogenesis of the breast in subgroups of breast tumours. In this context we have investigated the subgroup of somatostatin receptor positive breast tumours. In this chapter the structure, expression, function and distribution of somatostatin receptors will be discussed.

Somatostatin

Somatostatin was originally identified as a cyclic tetradecapeptide with an inhibitory effect on hormone secretion. In man two somatostatins exist, SS-14 and SS-28; both derive from pro-somatostatin by alternative processing. Somatostatin is well conserved during evolution and even in protozoa somatostatin-like peptides have been found. Somatostatins are widely expressed throughout the human body. It is present in the CNS where it acts as an inhibitory neurotransmitter. In the pituitary it acts as a neuro-hormone to inhibit growth hormone release. Additionally, it acts as an inhibitor of both endo- and exocrine secretory processes in a number of organs like the gastrointestinal tract and the endo- and exocrine pancreas[8,9].

The antiproliferative effect of somatostatin.

In the context of dysregulated cell growth as occurs in cancer cells, the antiproliferative effect of somatostatin and its analogues is particularly interesting. Administration of somatostatin or somatostatin analogues, resulted in regression or stabilization of the tumour in 50% of human growth hormone producing pituitary adenomas[10-12], in some endocrine pancreatic tumours[12], metastatic carcinoids[13], and medullary thyroid carcinomas[14]. Except for the

pituitary adenomas this effect was transient, suggesting either selection for and outgrowth of SSTR-negative cells or down regulation of SSTRs[15]. No effect of therapy with somatostatin analogues was observed on tumour size in heavily pretreated breast cancer patients[16], small cell lung carcinoma[17], colonic, gastric and pancreatic adenocarcinomas[18] and in a small series of nonendocrine tumours[19], despite suppressed insulin-like growth factor 1 (IGF-1) levels.

Extensive studies have been carried out in experimental tumour models in animals. In chemically induced hamster pancreatic tumours both the number and the size of the tumours reduced upon administration of somatostatin analogues[20-23]. A suppression of tumour growth was also observed in other transplantable tumours: rat Dunning R-3327 prostatic carcinoma[24-26], DHD/K12 colon carcinoma[27], 7315a pituitary adenoma[28], mouse Dunn osteosarcoma[29] and MXT mammary carcinoma[30] and human small cell lung carcinoma cell lines[31]. These tumours often show a slower growth rate than the tumours in control animals. No decrease in tumour size was observed. No inhibitory growth effect was observed in the SSTR-negative rat DMBA mammary carcinoma[32].

A direct growth inhibiting effect was observed in a number of in vitro cultured tumour cell lines. It completely inhibited the EGF-induced centrosome separation and DNA synthesis in Gerbil fibroma and HeLa cells[33]. Also the EGF or serum induced growth of a small intestinal crypt cell line IEC-6[34], the pancreatic cell lines MIA PaCa-2 and AR4-2J[35-37], the breast cancer cell lines MCF-7, ZR-75-1, T47D and MBA-MB-463[38-40], the pituitary cell line 7315b[41] and small cell lung carcinoma cell line NCI-69[31] was retarded 20-100% by somatostatin or its analogues. In contrast to these inhibitory effects, growth stimulation by somatostatin has been shown in meningioma cells and activated lymphocytes[42,43].

Mechanisms mediating the antiproliferative effect.

Several mechanisms have been implicated, that mediate this growth regulatory effect of somatostatin:
Indirect, by the inhibition of the effects of growth stimulating hormones and growth factors, like growth hormone, insulin, gastrointestinal hormones and IGF-1[44]. Other ways in which somatostatin may act indirectly are by inhibition of angiogenesis[45,46] and by the stimulation of Kupffer cells which prevent homing and therefore the outgrowth of tumour cells[47].

However, *in-vitro* studies suggest that somatostatin and its analogues exert a direct growth inhibitory effect on tumour cells via somatostatin receptors on the cell surface. Octreotide, a synthetic somatostatin analog, inhibits the growth of AR4-2J rat acinar pancreatic cells that contain somatostatin receptors[37]. It did not suppress basal cellular cAMP levels, but suppressed the vaso-intestinal peptide-induced raise of cAMP. Furthermore pertussis toxin does not reduce the growth inhibitory effect of Octreotide although it abolishes the antisecretory effect of somatostatin[37]. Studies by Hofland, *et al.* on the growth of cultured tumour cells derived from a transplantable rat prolactinoma showed that the antisecretory effects of somatostatin can occur in conditions where the antiproliferative effect is not seen[48]. The two are not always linked, although both effects depend on the presence of somatostatin receptors.

Finally, histological studies have suggested that somatostatin and its analogues enhance programmed cell death or apoptosis through an as yet unknown mechanism[20,21,30]. Somatostatin has both direct and indirect growth inhibitory effects on normal and tumour cells. Several of these effects have been shown to be mediated by somatostatin receptors. Unravelling the mechanisms by which these receptors mediate these effects may learn us a lot about tumour growth inhibition and probably put us on the trail towards the development of new antiproliferative drugs.

Somatostatin receptors

Somatostatin exerts its action by binding to a somatostatin receptor (SSTR). SSTRs are membrane bound proteins that can be demonstrated in biochemical binding assays or by *in-vitro* and *in-vivo* autoradiography using radiolabeled somatostatin or somatostatin analogs as a probe. Using various methods, SSTRs were demonstrated in the brain, the anterior pituitary, the neuroendocrine cells of the pancreas and intestines, and in many tumours that derive from these tissues. Specific somatostatin binding has also been demonstrated in some tumours that derive from tissues in which no somatostatin binding could be detected, like breast, colon and ovary cancers[15]. At present it is not known whether all these tumours derive from cells belonging to the dispersed neuroendocrine system. If this is the case, SSTR expression might be part of the normal phenotype of the cell. Alternatively, SSTR expression could be the result of a changed differentiation pattern of these tumour cells.

In the past autoradiographic and biochemical experiments suggested the existence of several different SSTR subtypes[49-64]. The molecular cloning of five human somatostatin receptors has confirmed the existence of this receptor family. The different members SSTR1-5, show differences in affinity for somatostatin or somatostatin analogues[55,56], a tissue specific expression and different effector pathways (Table II). These receptors are characterized by seven transmembrane domains and belong to the class of G-protein coupled receptors. Several SSTR-linked intracellular effector systems have been identified, which add to the pleiotropy of the system[54,64].

Receptor activation is associated with an immediate reduction of cAMP and Ca^{2+} levels in the cell or results in the induction of tyrosine phosphatases.

The reduction of cAMP levels is caused by inhibition of the enzyme adenylate cyclase[65-67]. Both a cAMP-independent mechanism which reduces Ca^{2+} influx by increasing membrane conductance to K^+ [68-70], and a direct inhibition of Ca^{2+} channels, result in lower intracellular Ca^{2+} levels[69,70]. These effects have been observed after binding of somatostatin to the somatostatin receptor.

Induction of tyrosine phosphatase by an as yet unknown mechanism also involves coupling of a SSTR to a G-protein[35,36,71-73]. These tyrosine phosphatases reverse the action of tyrosine kinases such as the epidermal growth factor receptor and especially this pathway has been implicated in the inhibition of tumour growth by somatostatin or its analogues[35,44]. Several oncogenes also function as tyrosine kinase. In this sense the SSTR could act as an anti-oncogene or tumour suppressor gene.

Somatostatin receptors in breast cancer

Somatostatin receptors have been identified in 8-75% of primary breast tumours. Fekete, *et al.* detected binding of somatostatin in 36% of 500 breast cancer biopsy homogenates[74]. Using autoradiography, Reubi and Torhorst[75] and Papotti, *et al.*[76] detected specific somatostatin analog binding in 17%-20% of primary breast tumours. In a more recent study where large tumour sections were used, Reubi, *et al.* detected somatostatin analog binding in 46% of primary breast tumours[77]. In our study described in chapters III.1 and III.2 a much higher incidence of 67-80% of SSTR expression in breast cancer was detected using *in-vivo* and *in-vitro* autoradiography, respectively.

As will be discussed in chapter III.1 part of the discrepancy in incidence could be explained by the detection of heterogeneous receptor expression in 30-50% of the tumours using *in-vitro* autoradiography. Earlier studies have not shown heterogeneous expression. This could be attributable to the size of the tumour sections used in the different studies.

Some data on the associations of SSTR expression and other clinical and pathological parameters of breast cancer are available: A correlation between SSTR and oestrogen receptor expression and an inverse correlation between SSTR expression and EGF receptor expression have been reported, suggesting a favourable prognosis[75,77]. This is also suggested by the correlation between somatostatin receptor expression and neuroendocrine differentiation in a series of primary breast tumours[76], since overexpression of the *c-ERBB2* oncogene,

TABLE II: MOLECULAR CHARACTERISTICS AND TISSUE EXPRESSION OF FIVE CLONED HUMAN SOMATOSTATIN RECEPTORS.

	SSTR1[57]	SSTR2[57]	SSTR3[58]	SSTR4[58-61]	SSTR5[56,58,63]
chromosome	14q13	17q23-24	22q13	20p11	?
amin acids	391	369	418	388	364
mRNA (kB)	4.5	2.5/8.5	4.8	4.6	2.6
tissue distribution	brain pancreas pituitary lung	brain kidney pancreas	brain pancreas	brain	pituitary
second messenger	G-protein	G-protein	G-protein adenylylcyclase	G-protein adenylylcyclase	G-protein

implicated in aggressive tumours, does not occur in neuroendocrine differentiated breast tumours[78].

In conclusion the impact of SSTR receptor expression on tumour characteristics and the course of the disease is not yet clear. Circumstantial evidence suggests an association between SSTR expression and parameters of less aggressive behaviour. More data on the tumour and patient characteristics of SSTR-positive tumours are presented and discussed in chapter III.

Somatostatin receptors and neuroendocrine differentiation in breast cancer.

Breast carcinomas with characteristics of neuroendocrine differentiation have been observed by several authors. Depending on the neuro-endocrine marker used, the reported incidence varies between 5 and 50 percent. The following methods and markers can be used to detect neuro-endocrine differentiation:

1. Electron microscopy: visualization of cytoplasmic, small-sized, dense-core, membrane bound secretory granules located at the vascular pole of the cell is widely accepted as the golden standard of detection of neuroendocrine differentiation. The method is labourious and hardly feasible for routine screening of series of tissue samples.

Therefore other easier to do but more difficult to interpret parameters have been used.

2. Morphology: monotonous solid growth in nests or sheets, granular eosinophilic cytoplasm, round or ovoid nuclei with only a slight variation in size and lack of glandular differentiation are morphologically characteristic for neuroendocrine differentiation.

3. Argyrophilia assessed by the Grimelius silver impregnation procedure[79] is a marker for the presence of secretory granules in the cells. Grimelius positivity has been reported in 5-50% of breast tumours[80-84]. This wide range results from the use of different methods and different criteria for positive cells. Azzopardi has proposed that only black granules should be considered positive. Using this criterion he[83] and others[85,86] including ourselves detect Grimelius positivity in 5-8% of the breast carcinomas.

4. Immunohistochemical reactions with several antibodies raised against antigens or hormones have been found in neuroendocrine cells. The ones that are routinely used are antibodies against neuron-specific enolase (NSE), chromogranin A and B (ChrA and ChrB), synaptophysin and neuroendocrine hormones.

NSE positivity has been found in 25 to 50% of tumours. The specificity of this marker is disputed. Nesland, *et al.* who detected positivity in 16 of 42 primary tumours including all 11 tumours in their series that produced hormones, conclude that NSE is the best marker for screening of breast tumours for neuroendocrine differentiation[87], whereas Papotti, *et al.* who found a similar percentage of NSE positivity in their series doubt that NSE antiserum is a reliable marker of neuroendocrine differentiation[76]. Chromogranin A and B are proteins that are part of the membrane of the dense-core vesicles seen in electron microscopy. Synaptophysin is a component of the clear vesicles of the pre-synaptic type[76].

On the basis of the correlation of these markers and the Grimelius silver impregnation with ultrastructural markers of neuroendocrine differentiation[86], we accepted argyrophylia, presence of chromogranin A and/or B, and of synaptophysin as reliable histochemical markers of neuroendocrine differentiation.

The origin of neuroendocrine differentiated breast carcinomas is still controversial. The dispute is about whether these carcinomas derive from the neuroendocrine cells that can be found in normal breast tissue. Bussolati observed chromogranin positive cells dispersed throughout normal breast tissue and supposed that they may give rise to these tumours[88]. On the other hand Wilander noted that in the breast cancer samples they had previously classified as Grimelius positive, the positive endocrine cells are a minority surrounded by Grimelius negative cells[85]. This implicates either loss of neuroendocrine characteristics by most of the cells in the case the tumours derive from neuroendocrine cells, or some cells could have gained these neuroendocrine characteristics. Interestingly, Capella, *et al.* observed so-called amphicrine cells

that show both exo- and endocrine (mucin and neurosecretory granules) characteristics at the ultrastructural level[89]. This observation supports the possibility that one stem cell could give rise to all types of breast carcinoma. A similar conclusion has been put forward by van Laarhoven, *et al.* who add that thus no different treatment is required for neuroendocrine tumours[90].

Nesland, *et al.* suggest that neuroendocrine systems are switched off in tumours that express the *c-erbB2* oncogene[78]. They tested 90 primary breast tumours immunohistochemically and found that NSE and c-erbB2 expression are mutually exclusive. In this model they imply that overexpression of c-erbB2 stimulates intracellular second messenger systems to such an extent that stimulation of peptide hormone receptors is no longer necessary: auto- and paracrine mechanisms are switched off. The question of the origin of neuroendocrine breast tumours has not yet been answered. Studies at the genetic level aimed at the identification of common or subgroup specific genetic alterations could help to find the answer. Chapter III.1 contains our survey of this problem.

Several authors have investigated the biological and clinical characteristics of neuroendocrine differentiated breast tumours. Capella, *et al.* found no correlation between ultrastructural features and histological, clinical or pathological data[89]. Monaghan and Roberts detected no correlation between histology and NSE positivity[91]. Azzopardi, *et al.* conclude that argyrophylic carcinomas form a wide range of morphology, histochemistry and prognosis[83]. Nesland, *et al.* reported that a subgroup of NSE positive tumours is aneuploid[92]. Aneuploid tumours of grade I were primarily found in the NSE positive subgroup. Wilander observed that Grimelius positivity correlated with diploid DNA values and neurohormone production in these tumours[85]. Thus, again a heterogeneous spectrum is found. As mentioned earlier Papotti reported a strong correlation between high density SSTR expression and neuroendocrine differentiation[86]. They observed that neuroendocrine differentiation tends to occur in older patients. In chapter III.1 we discuss the results of our investigation of the association between SSTR expression and neuroendocrine differentiation in primary breast tumours.

II.3: BREAST CANCER IS A GENETIC DISEASE

Introduction

Disease can be regarded as a result of an interaction between environmental and constitutional factors. This is also the case with cancer. Environmental factors that can cause cancer are for instance UV-radiation, chemical compounds and certain viruses. These factors share the capability of inducing damage to DNA and by that creating mutations in the DNA sequence. On the other side is the observation that some people are more prone to develop a cancer than others, eg members of certain cancer-prone families. It has been suggested that this is the result of the propagation in the germline of a factor that alone does not cause disease but that may increase the susceptibility of the carrier to the development of disease. Such a mechanism has been proved in members of Li-Fraumeni families who carry a mutation in the P53 tumour suppressor gene and have a high probability of developing breast cancer, leukaemia and other cancers. A genetic basis is also clear at the cellular level: A tumour arises from one cell that acquires the malignant genotype and passed it on to her daughter cells. In the last decennia much has become clear about the type of DNA damage that is involved in carcinogenesis and the genes that are involved in this process. It started with Boveri who, early in this century said that the development of cancer was accompanied by specific chromosomal changes[93]. A classic example is chronic myeloid leukaemia, which is characterized by a specific chromosomal aberration: t(9;22), the Philadelphia translocation[94]. Subsequent studies revealed that as a result of this translocation the c-ABL oncogene moved to the BCR gene on chromosome 22 giving rise to a new fusion protein that has in-vitro oncogenic activity and induces a myeloproliferative syndrome in mice that is closely related to chronic myeloid leukaemia[95,96]. Oncogenes are present in all cells of the body and get transforming capacity once they have been activated. Oncogene activation can be induced by several mechanisms, like translocation as in chronic myeloid

leukaemia, but also by overexpression or ectopic expression as a result of gene mutation or gene amplification. The latter mechanism is fairly easy to detect and is frequently observed in breast cancer.

A second class of cancer genes has emerged in the 1980's, the tumour suppressor genes. These genes normally prevent tumour growth and both copies of the gene in a cell have to be inactivated for tumour growth to occur, for instance by point mutations or deletions. Inheritance of one mutated allele can predispose family members to cancer growth. The tumour suppressor genes have therefore been implicated in both sporadic and familial cancer.

The existence of tumour suppressor genes was originally inferred from underrepresentation of chromosomes or parts of chromosomes as a result of inadequate chromosome disjunction that has frequently been observed in tumour DNA[97]. This may lead to expression of recessive phenotypes due to for instance loss of a tumour suppressor gene[98,99], or alterations in gene dosage in tumour cells[100,101].

The retinoblastoma tumour suppressor gene (*RB*) was the first identified tumour suppressor gene, and has become the paradigm of this class of genes. The first steps to its identification were clinical observations: Patients who have a hereditary predisposition for retinoblastoma usually get more than one tumour and at a younger age than patients without this predisposition who usually have only one tumour. Knudson presented a statistical model that explained these differences in incidence[102]. He postulated a tumour suppressor gene that would allow tumour growth in a retina cell only when both alleles of the gene in that particular cell were inactivated by a mutation. In hereditary retinoblastoma one mutated allele is inherited and is thus present in all retina cells. A tumour can arise in any of these cells when a second (somatic) mutation inactivates the other allele. In sporadic retinoblastomas two somatic mutations have to take place in the same retina cell. The chance for this to occur is much smaller and therefore usually only one tumour is seen in sporadic cases of retinoblastoma. This theory was validated by the molecular cloning of the retinoblastoma gene in 1986[103]. Several additional tumour suppressor genes, also in breast cancer have since been identified (Table III). Not all these genes act analogous to the

RB gene. An example is the P53 gene, where in some tumour types, the mutated allele has been shown to have a dominant negative effect over the wild type gene. There one mutation abolishes the tumour suppressor function of the gene.

TABLE III: examples of tumour suppressor genes in human cancers.

Gene	Cancer type	hereditary syndrome
APC	colon	Familial adenomatous polyposis
DCC	colon	-
NF1	neurofibromatosis	NF1
NF2	meningiomas schwannomas	NF2
P53	various cancers	Li Fraumeni
RB	retinoblastoma	retinoblastoma
VHL	kidney	Von Hippel Lindau
WT1	nephroblastoma	Wilms tumour

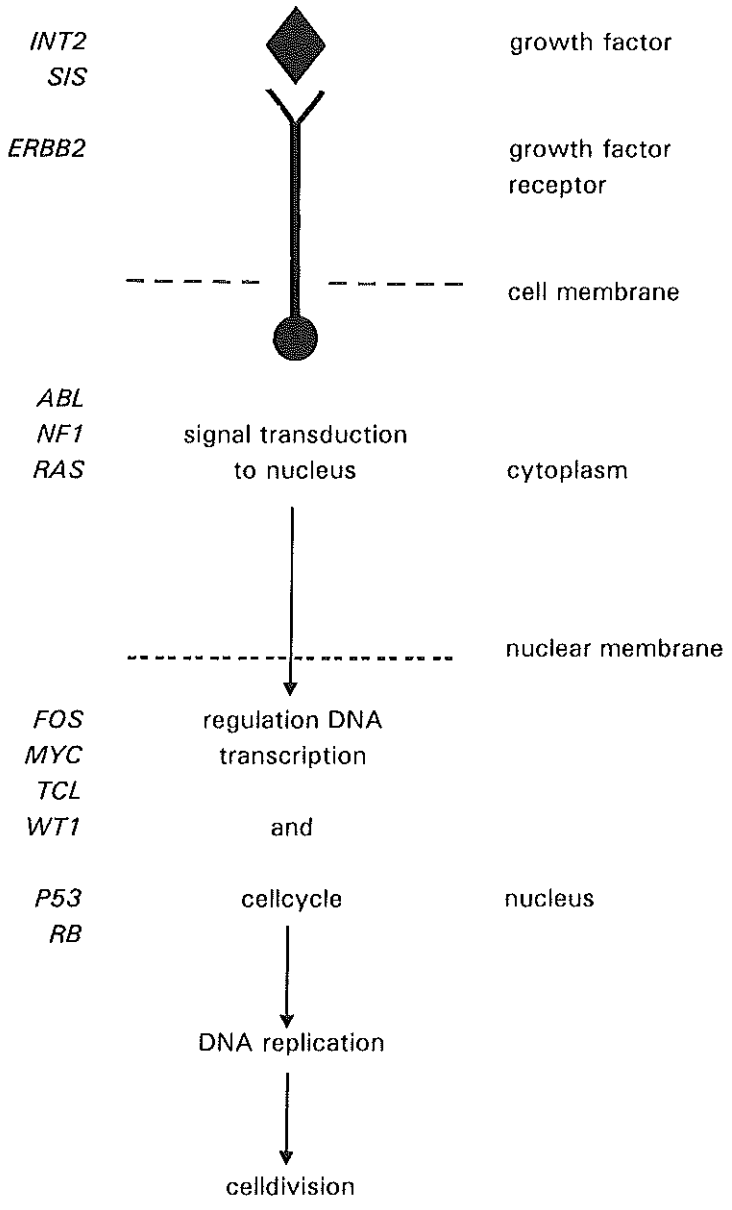
A third class of genes involved in carcinogenesis are the genes that encode proteins with a function in the repair of (carcinogenic) mutations in the DNA. Failure of these mechanisms enhances the process of carcinogenesis. They will be further discussed later.

Several different genetic alterations are usually observed in cells from solid cancers, like breast cancers. These alterations include both oncogenes and tumour suppressor genes. Vogelstein proposed a multistep model for colon carcinoma in which the accumulation and cooperation of these alterations, possibly in a certain sequence ultimately lead to the full carcinogenic genotype[104]. It is very likely that an analogous model will emerge for breast cancer. The phenotypic heterogeneity of breast cancer might then probably be explained by different sets of genetic alterations.

Function of oncogenes and tumour suppressor genes

Cells depend on signals from outside for the regulation of their growth and differentiation. These signals contact the cells through receptors after which they are transduced via second messenger systems to the nucleus where the effect is generated. Cancer can be regarded as a disease in which the cellular responses to these signals are disrupted. It is therefore not a surprise that the products of genes that are involved in the regulation of growth and differentiation also play a role in carcinogenesis. The function of several oncogenes and tumour suppressor genes has been elucidated. As shown in figure 1 they are indeed present at all stages in the regulation of cell growth and differentiation.

FIGURE 1: function of oncogenes and tumour suppressor genes.



Genetic alterations in breast cancer.

In breast cancer genetic changes such as in chromosome number or structure, alterations in oncogenes and tumour suppressor genes have been observed. In this chapter we will review these genetic changes and discuss their linkage with clinicopathological parameters as step towards the identification of genetically homogeneous subgroups of breast tumours.

Chromosome content: DNA ploidy

A crude method to detect genetic changes is the assessment of the DNA content of tumour cells. Series of primary breast cancers show a nonrandom bimodal distribution of DNA indices with peaks in the near-diploid and the hypotetraploid region. Approximately 70% of the breast tumours have an aneuploid stemline and at least ten to 20 percent show evidence of more than one aneuploid stemline[105]. Univariate analysis of the prognostic significance of DNA ploidy demonstrated that aneuploidy ranked third only behind nodal status and tumour size. This prognostic significance diminished, however, in multivariate analysis with most clinico-pathological variables. Apparently, more subtle genetic methods are required to be able to link genetic changes to clinical and pathological variables. An interesting result from DNA ploidy analysis concerning breast cancer progression was put forward by Bonsing and coworkers, who reported that most DNA tumour stem lines recurred in lymphnode (55%) and distant metastasis (59%)[106]. However, the stem lines in distant metastases often differed from those in the lymphnode metastases (61%). This is in contrast to the traditional idea that a tumour metastasises first to the regional lymphnodes and from there to distant loci. This emphasizes the need for more sensitive screening methods for distant metastases at the time of diagnosis (see chapter III.2).

Cytogenetics of breast cancer

To further elucidate the genetic changes in breast cancer, the number and structure of the chromosomes of breast cancer cells have been investigated. Unfortunately, banded karyotypes of breast tumours cells are scarce. The main reason being technical difficulties in culturing breast cancer cells. Rapid overgrowth by fibroblasts and the admixture of preexistent normal epithelium in the tumour often results in the detection of normal karyotypes, which most likely derive from the noncancerous cells. Despite these problems a few reports on karyotype analysis of breast cancer cells are available.

The reported tumour cell karyotypes are usually complex and show both structural and numerical alterations. Chromosome one is most frequently involved in both structural (translocations, marker formation) and numerical changes[106-116]. Other chromosomes that are frequently involved in structural aberrations are chromosomes 3, 6, 7, 11, 15, 16, 17, 18, 19, 20 and 21. Cytogenetic markers of gene amplification: homogeneously staining regions (HSR) and double minutes (DM), have been observed regularly. Specific involvement of chromosomes 8p in these markers has been reported[117,118], but the gene(s) involved have not yet been identified[117]. Several oncogenes that are frequently amplified in breast cancer, like *INT2*, *MYC*, *NEU* and *FES*, were investigated as candidate genes but were not amplified in tumours that contained HSR. In one study five out of 72 primary breast tumours contained HSRs and in two of these also an amplified NEU oncogene was found[108]. Mostly the origin of 8p homogeneously staining regions remains obscure.
Loss of chromosomes is reported most frequently for chromosomes 17[108], 8, 13[111,119], 16[111], 11q, 16q 1p, 8p, 13q, 6q, 17p[113]. Alterations of 1q11-22 have been reported in lymphocytes of breast cancer patients (both sporadic and familial) and also in the lymphocytes of some predisposed family members. The 1q rearrangement was therefore proposed as a primary lesion[107]. Alterations at 16q22 have also been put forward as the primary event in breast cancer[106].
These results clearly show the complexity and the heterogeneity of the

molecular events taking place in breast cancer. Striking is also the discrepancy between the molecular data that implicate a frequent involvement of NEU, MYC and INT2 oncogenes and the cytogenetic data where no amplification of these oncogenes was observed on the HSR in breast tumours. This could say that other yet unidentified genes, are involved in breast cancer.

Oncogene activations in breast cancer

Oncogene amplification is a frequent phenomenon in breast cancer. The oncogenes involved are the *NEU*, *INT2* and *C-MYC* oncogenes. Other oncogenes, like *BCL1*, *HRAS*, *RAF1*, *CMYB* and *CERBA2* have also been implicated in breast cancer, but in these cases, amplification is not always the mechanism of oncogene activation, but also loss or change of activity as the result of deletion has been observed in these loci.

The *NEU* oncogene is by far the most studied oncogene in breast cancer. Amplification is observed in up to 33% of primary breast tumours and correlates well with expression of the *NEU* protein[120-122]. Correlations of *NEU* amplification or *NEU* protein overexpression with tumour and patient characteristics have also been investigated. A correlation has been reported with lower tumour differentiation grade[121,123-129], lymphnode involvement[121,125,130,131], and absence of steroid receptors[124,125,129,131]. A correlation of *NEU* activation with reduced survival or disease free interval has been reported both in all breast tumours with an activated *NEU* oncogene[127,128,132-134], and in subgroups of these tumours: node positive[125,131,135], high grade[136] or advanced stage tumours[137]. These results suggest that *NEU* activation is a marker of advanced or aggressive disease. However, this suggestion is weakened by the results of other studies: Absence of a correlation with node positivity[127,138], presence of steroid receptors[127,139,140], reduced disease free interval or survival[125,126,131,133,135,137,138,141-145]. Two reports mention *NEU* amplification as independent predictor of clinical outcome in a multivariate analysis[131,146].

Interestingly Berns, *et al.* recently reported that *NEU* amplification did not affect overall survival because patients with an amplified *NEU* oncogene in their tumours showed a good response on chemotherapy, but responded less on endocrine therapy after disease recurrence, resulting in no difference in survival[147]. This observation is in line with the correlation between *NEU* overexpression and the absence of steroid receptors and high grade, fast growing tumours and with the disputed influence on survival. It can also explain the decreased survival in subgroups as those subgroups are characterized by established markers of worse prognosis, and this thus reflects the scarcity of data of *NEU* as an independent prognostic factor. In conclusion the *NEU* oncogene may be important in selecting patients for therapy strategies, rather than as an indicator of prognosis.

INT2 amplification has been found in up to 23% of primary breast tumours[148-152]. *INT2* is usually amplified in an amplicon that also contains the *HST* and *BCL1* oncogenes. It is as yet not clear whether any of these genes is indeed involved in breast cancer. This amplification has been associated with local recurrence or distal metastases[150], lymphnode involvement[141], oestrogen receptor positivity and younger age of the patient[152]. Furthermore, an association with poor tumour differentiation, shortened relapse free interval and overall survival has been reported[153]. This association was also observed by Schuuring and coworkers (1992) who investigated the amplification of the entire 11q13 region[154]. *INT2* amplification has also been associated with amplifications of oncogenes at other loci predominantly *NEU* and *CMYC*[153]. However, an inverse correlation of *INT2* amplification with *NEU* amplification has also been reported[148].

CMYC amplification has been observed in 4-32% of primary breast carcinomas[124,130,132,145,153,155]. It has been correlated to poor tumour differentiation[153], inflammatory disease[124], absence of progesterone receptors[145], older age at onset[155], rapid disease recurrence[156], and poorer short term prognosis[132,147,157]. Amplification is one mechanism that can lead to

overexpression of the gene. This was shown most strikingly in a study by Geurin, *et al.* who reported *c-MYC* amplification in 6/100 tumours, whereas elevated RNA levels were detected in 44/98 (45%) of these tumours[130].
At the RNA level high expression correlated with lymphnode involvement[130], while at the protein level this association was not found[158]. High MYC protein levels have been correlated with more widespread disease[159], and with a better differentiation of the tumours[158]. However, in these studies it was not predictive of survival or disease free interval. The origin of these discrepancies may be differences in samples of heterogeneous tumours or perhaps the result of transient gene expression.

Tumour suppressor genes and breast cancer.

A widely used method to gain indirect evidence for the involvement and localization of a tumour suppressor gene is screening for allelic imbalance. This approach compares the constitutional genotype at a given locus with that of the tumour. Using this method loss of alleles (loss of heterozygosity) but also duplication of alleles can be detected[160]. Table IV summarizes data available on allelic imbalance in human primary breast tumours. Thirteen chromosome arms are frequently involved in loss of heterozygosity (LOH) implicating the presence of a tumour suppressor gene at these sites. Two of such genes have been identified: the *RB* and the *P53* tumour suppressor genes on chromosomes 13q and 17p, respectively. These genes have also been implicated in breast carcinogenesis.
Series of breast tumours show a heterogeneous pattern of allelic imbalance. It is not fully understood whether this reflects different stages of tumour development in which all tumours sequentially accumulate losses at all these sites, or that alternatively different subgroups of breast tumours acquire different gene losses. A third possibility is that several genetic alterations are required for tumour progression, whatever the kind of alteration. This is supported by the association of many allelic imbalances with imbalance at other

sites, also suggesting that these are late events in carcinogenesis[175]. Devilee, *et al.* say that absence of allelic losses correlates with absence of metastasis[176].

TABLE IV: Loci with frequent allelic imbalance in breast cancer

chromosome locus	frequency (%)	reference
1p32	37	161
1p34	63	162
1p36	41 (all)	163
	60 (familial)	
1q21-q23	26	164
3p14-p23	46	165
	34	160
6q	50	160
8q	40	160
11p15	21	165
	20	166
	21	167
13q12-q134	25	165
	28	160
	21	168
15q	37	160
16q24	40	160
	45	168
	63	169
17p13.3	67	165
	61	167
	56	168
17q23-25	35	170
	38	171
	31	160
18q	38	172
	25-69	171
22q	38	173
	24	174

However, there are also indications that some order is present in the genetics of breast cancer and that it is not simply an accumulation of alterations that cause and lead to progression of breast cancer. Allelic imbalance at chromosome 16q and 1q, is not associated with imbalance at other loci, suggesting that these events occur at an early stage in carcinogenesis[175]. Furthermore several authors have reported losses of specific chromosome loci in subsets of breast tumours. Devilee, *et al.* find loss of heterozygosity of chromosome arm 17p in all four lobular carcinomas included in this study[176]. In contrast Larsson, *et al.*, detect 17p loss predominantly in ductal carcinomas and loss of chromosome 22q sequences in lobular carcinomas[174]. These results implicate that the investigation of subgroups of breast carcinomas is indeed worthwhile.

The retinoblastoma tumour suppressor gene and breast cancer

The retinoblastoma tumour suppressor (*RB*) gene was originally identified as the tumour suppressor gene involved in retinoblastoma. However, this tumour suppressor gene has been implicated in the etiology of many other tumours, as well. Deletions are common in osteosarcomas, a tumour which was already known as a frequently occurring second tumour after retinoblastoma. However, alterations have also been found in non-retinoblastoma related tumours like lung carcinoma, prostate cancer and breast cancer. Proof for *RB* gene involvement in tumourigenesis comes from experiments in which copies of the wild type *RB* gene were reintroduced in cells that contained only mutated endogenous *RB* alleles[177]. After introduction of wild type *RB* gene copies the cells lost several of their malignant characteristics.

In breast cancer alterations in the retinoblastoma gene have been observed in 22-25% of breast cancer cell lines[178,179] and 13-75% of primary breast tumours[179-181]. Using immunohistochemical techniques loss of *RB* protein expression is detected in 29% of the primary breast tumours[180]. All these tumours had detectable *RB* gene alterations. Trudel, *et al.* recently reported a

complete loss of *RB* expression in 19% and heterogeneous expression in 28% of the primary breast tumours[182]. In that study also increased expression was detected in some tumours with high nuclear grade.

Several reports suggest a role of *RB* gene alterations in advanced disease. Varley, *et al.* reported that loss of the *RB* gene is more frequent in poorly differentiated and more widespread tumours[180]. Trudel, *et al.* report a weak ($p=0.045$) association between immunohistochemical presence of RB protein with grade 3 tumours[182]. No reports on the impact of *RB* gene alterations on disease-free interval and overall survival have been published so far. In chapters IV.1 and VII the association between *RB* gene alterations with tumour and patient characteristics and the impact of these alterations on the course of the disease is discussed.

Loss of heterozygosity of chromosome 17p13, the *P53* gene and breast cancer.

The P53 gene is located at chromosome 17p13.1. It has been shown to be the predisposing gene to the Li-Fraumeni syndrome, a hereditary cancer syndrome that besides breast cancer show a predisposition to soft-tissue sarcomas and cancer of the brain and lung[183].

Also in sporadic breast cancers alterations in the *P53* gene have been demonstrated. Loss of heterozygosity of chromosome 17p has been shown in about 60% of the investigated breast cancers[167,176] and 20 to 40% of the breast tumours have been shown to contain mutations in the *P53* gene[184-186]. In breast cancer allelic imbalance has been correlated with late stage, high grade and metastatic spread[186,187]. Sato, *et al.* reported that loss of both the RB and the *P53* gene correlates with a more malignant phenotype[168]. Although a correlation between the *P53* mutations and loss of heterozygosity of chromosome 17p has not been observed in each study, this suggests involvement of the *P53* gene in breast cancer by inactivation of the gene. This assumption is supported by the reversal of the malignant phenotype of breast cancer cells upon introduction of the wild type *P53* gene into these cells.

However, in the majority of tumours containing a mutated *P53* allele, this mutant allele is overexpressed[189], and can abolish the function of the remaining wild type allele. This dominant negative effect is different from the classic tumour suppressor gene dogma in which all wild type alleles have to lose their function.

Intracellular levels of P53 rise in a response to DNA damaging agents[190,191] and high levels of P53 protein have been shown to block G1 to S phase transition. Possibly P53 is involved in sensing DNA damage giving the cell time for DNA repair before DNA replication[190-194]. Malfunction of the *P53* gene could hamper DNA repair and enhance carcinogenesis.

Genetic predisposition to breast cancer.

A third strategy to delineate the genetic heterogeneity of breast cancer is to identify persons that are clearly predisposed to breast cancer and to search for the predisposing genetic alteration in these individuals.
Usually genetic linkage analysis in breast cancer families is used to identify the predisposing gene defects. Familial aggregation is observed in approximately 15 % of all breast cancers[195]. In about a third of these familial cases the breast cancer is really hereditary because there is a mendelian pattern of inheritance. This pattern is often autosomal dominant with incomplete penetrance. In these families breast cancer is often diagnosed at a significantly younger age, is more often bilateral, and specific associations with other tumours in particular cancer-prone syndromes have been described (see table V).

TABLE V: Heterogeneity in hereditary breast cancer

Site specific breast cancer[196]
Cowden's disease[197]
Breast-Ovary cancer syndrome[198]
Breast-Gastrointestinal cancer syndrome[199]
Li-Fraumeni syndrome[183]

This table is another example of breast cancer heterogeneity. Are the different syndromes caused by different genotypic alterations? There is increasing evidence that this is indeed the case. In the Li-Fraumeni syndrome breast cancer is associated with childhood sarcoma, brain tumours and adrenocortical carcinomas[183]. The disease is linked to germ-line mutations in the P53 gene[200]. In other families with a predisposition to breast and ovary cancer, the disease has been linked to a gene on chromosome 17q12-21, *BRCA1*[198,201,202]. This, as yet unidentified gene is also involved in 50% of the families that show a predisposition to early-onset breast cancer (age at onset under 47 years)[201]. Using both deletion- and linkage mapping the localization of the *BRCA1* gene on chromosome 17q22 has been narrowed down to approximately 500 kb. From this region the first candidate gene has been isolated[203].
Identification of the *BRCA1* gene will have major clinical implications and will be used to identify women at high risk well before the disease is expressed. Recent estimates suggest that the predisposing allele of the *BRCA1* gene is carried by one in 200 women and imposes a greater than 85% chance of actually getting breast cancer on these carriers[201].
Yet other genes must predispose to breast cancer in the other half of the early onset breast cancer families and in the remaining breast cancer syndromes.

Another approach used to identify markers of genetic predisposition is a more mechanistic one. Cancer is the result of mutations in the DNA. Fortunately the body possesses mechanisms to repair the DNA damage before cell replication. Therefore these DNA repair mechanisms play a role in cancer prevention, and

defects in DNA repair mechanisms will lead to an increased incidence of cancer. When defects in DNA repair are inherited they can predispose to cancer. DNA repair mechanisms and the genes involved in DNA repair are thus obvious targets for research on carcinogenesis. The best known example is xeroderma pigmentosum. In this disease a defect in the excision repair mechanism makes the patients extremely sensitive to UV induced DNA damage and multiple skin cancers may develop in the UV exposed area's of the skin of these patients.

An association between DNA repair and breast cancer has been observed in ataxia telangiectasia (AT). This is an autosomal recessive condition that is characterized by cerebellar ataxia, oculocutaneous telangiectasia, a hypersensitivity to ionizing radiation and an increased susceptibility to cancer[204]. Epidemiological research has suggested that heterozygotes for a mutated AT gene have an increased risk of developing cancer, in particular breast cancer[205-208]. The relative risk for heterozygotes has been estimated at 6.8, with a carrier frequency of 1% of the population. These data implicate that at least 10% of all breast cancers may occur in AT carriers. As a possible explanation, an increased susceptibility in these gene carriers to ionizing radiation has been suggested[206]. However, these data have been disputed[209-215]. The mechanism by which AT heterozygosity leads to increased DNA mutability and cancer has not yet been completely resolved. Linkage of the AT locus to familial breast cancer has been excluded[216].
Different DNA repair mechanisms are involved in hereditary-non-polyposis-coli (HNPPC). In this disease a type of genetic instability had been observed that, in analogy to similar defects in bacterial model systems, was supposed to be the result of defective DNA-mismatch repair. The involved gene, *MSH2*, appeared to be the human homolog of a previously cloned bacterial DNA-mismatch repair gene[217]. Similar genetic instability has been observed in about 8% of breast cancers[218].

Interestingly, Abrahams, *et al.* showed that skin cancers in xeroderma pigmentosum patients occur only in those patients which showed an enhanced

UV-induced activation of a different emergency DNA repair mechanism, named after the international emergency signal, the SOS repair mechanism[219]. Subsequently it was shown that this enhanced activation was also present in individuals from various hereditary cancer syndromes: retinoblastoma, Von Hippel Lindau, Neurofibromatosis type 1 and 2, polyposis coli and others[220]. This enhanced UV inducibility has also been demonstrated in a hereditary breast cancer syndrome: the P53 gene linked Li-Fraumeni syndrome (Abrahams, personal communication). This is suggestive of a role of these SOS repair mechanisms in carcinogenesis and provides further support for a more generalized involvement of DNA repair mechanisms in carcinogenesis and apparently not only by loss of function but also by gain of function of DNA repair mechanisms. In chapter V the possible involvement the SOS repair mechanism in a different type of hereditary breast cancer is under investigation.

II.4 INTRODUCTION TO THE EXPERIMENTAL WORK

The aim of the experimental work was to link genetic alterations in breast tumours to pathological characteristics of the tumour and to the course of the disease in the patient. The main difficulty is the heterogeneity of breast cancer from the molecular level up to the clinical level. To delineate this heterogeneity we investigated three subgroups of breast cancer: Primary breast cancers that express the somatostatin receptor, primary breast cancers with alterations in the retinoblastoma tumour suppressor gene and patients and family members from families that show a genetic predisposition to breast cancer.

In 1988, at the beginning of our experimental work, somatostatin receptor expression had been detected in approximately 20% of the breast tumours. As somatostatin is a marker of the neuroendocrine system, it may be assumed that those breast tumours that express this receptor compose a neuroendocrine subgroup of breast cancers. This neuroendocrine differentiation appeared functionally important since Foekens, *et al.* had shown that patients with a SSTR-positive breast tumours had a better prognosis than patients with a SSTR-negative tumour[221]. In other neuroendocrine tumours, like small cell lung cancer, alterations in the retinoblastoma tumour suppressor gene and specific oncogene amplifications had been detected. We chose this particular subgroup of somatostatin receptor positive breast tumours to investigate whether this subgroup of tumours had any subgroup specific genetic alterations in common. An obvious question was whether analogous genetic alterations had taken place in neuroendocrine breast tumours. Chapter III.1 describes our investigation of the incidence of somatostatin receptor expression and its correlations with alterations in the *RB* gene and some oncogene amplifications. In a parallel study an overlapping group of patients was investigated for the expression of the somatostatin receptor *in-vivo* (Chapter III.2). From these studies it became clear that somatostatin receptor expression is a frequently observed characteristic of primary breast cancers.
Somatostatin has a growth inhibitory effect on several types of cancer cells.

This effect is mediated by the somatostatin receptor. Defects in the somatostatin receptor or lower somatostatin receptor protein levels could enhance cell growth in a way similar to loss of a tumour suppressor gene. To investigate this possibility we attempted to clone these somatostatin receptor genes from SSTR-rich tissue RNA. During the course of our experiments Yamada, *et al* published the sequence of two of the SSTR genes[57]. These sequences were used in our studies of the localization and allelic imbalance of these somatostatin receptors in primary breast tumours (chapter III.4).

Our next step was to test if *RB* gene alterations were correlated with other tumour and patient characteristics and if these alterations have impact on the prognosis of the patient. In a collaborative study we investigated 96 tumours from the Dr Daniel den Hoed Cancer Centre for aberrations in the *RB* gene. From these tumours the pathological and the corresponding patient characteristics were available. These clinicopathological data were compared with the observed *RB* gene alterations to test if any correlations could be detected and if these alterations have an impact on the patients prognosis (chapter IV.1 and VII).

The relation between genes and breast cancer is perhaps most evident in hereditary breast cancer and it is tempting to search for the predisposing genes in these families. Identification of these genes would facilitate screening for women at risk, making early diagnosis more feasible and will lead to a higher overall survival of breast cancer. In chapter IV.2 a linkage analysis of a few Dutch hereditary breast cancer families with chromosome 17q markers is described.

A third type of cancer genes has gained attention in the studies of the development of solid tumours, the genes involved in DNA repair. They encode the proteins that compose the DNA repair mechanisms that are active in our cells. Recently it has become clear that aberrations in such a mechanism, the SOS repair mechanism can be observed in many cancer-prone syndromes, like Von Hippel Lindau disease and the Li-Fraumeni syndrome. This Li-Fraumeni syndrome is associated with a preponderance of breast cancer and some other malignancies and has been linked to a mutation in the *P53* gene. We investigated whether this change in DNA repair is also present in gene carriers

of a family with another type of hereditary breast cancer, linked to the *BRCA1* locus on chromosome 17 (chapter V.1).

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CHAPTER III: STUDIES OF SOMATOSTATIN RECEPTORS AND BREAST CANCER

III.1: SOMATOSTATIN RECEPTOR-POSITIVE PRIMARY BREAST TUMOURS: GENETIC, PATIENT AND TUMOUR CHARACTERISTICS.

International Journal of Cancer, 54: 357-362, 1993.

Int. J. Cancer: 54, 357–362 (1993)

UICC Publication of the International Union Against Cancer
Publication de l'Union Internationale Contre le Cancer

SOMATOSTATIN RECEPTOR-POSITIVE PRIMARY BREAST TUMORS: GENETIC, PATIENT AND TUMOR CHARACTERISTICS

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In a series of 87 primary breast tumors, somatostatin receptor (SSR) expression was detected by *in vitro* autoradiography in 58 tumors. In 41 tumors the SSR expression was homogeneous and in 17 it was heterogeneous. Although the tumors were not selected by the investigators upon entry in the study, examination of the tumor and patient characteristics showed that a pre-selection had taken place for small tumors. Eighty percent of the tumors were classified as stage pT1 or pT2 tumors. This small tumor size and the large size of the tumor sections used for autoradiography can explain the high incidence of somatostatin expression in our series. Forty-three of these tumors, 30 SSR-positive and 13 SSR-negative, were tested for morphological and (immuno)histochemical markers of neuroendocrine differentiation. Three SSR-positive tumors were also positive for 2 or more other markers of neuroendocrine differentiation, suggesting that neuroendocrine breast tumors and SSR-positive breast tumors are overlapping, but independent, subgroups of tumors. To test whether specific genetic alterations are associated with SSR-positive or SSR-negative breast tumors, we examined in a selected series of 47 SSR-positive and 32 SSR-negative breast tumors a number of known genetic markers by Southern blotting. Deletions or rearrangements of the retinoblastoma (*RB*) tumor-suppressor gene were observed in 5 SSR-positive and 5 SSR-negative tumors. In 4 SSR-positive and also in 4 SSR-negative tumors an amplification of the *neu* oncogene was observed. Amplifications of the *int*-2 oncogene were found in 2 SSR-positive and 1 SSR-negative breast tumor. In one SSR-positive tumor an amplification of the c-*myc* oncogene was observed and in another SSR-positive tumor a rearrangement of the L-*myc* oncogene was found.

The study of breast cancer carcinogenesis is complicated by the heterogeneity of the disease. One way of simplifying the matter is to classify these tumors into clinically relevant subgroups. There are indications that breast carcinomas which express the somatostatin receptor (SSR) are such a distinct subset. For example, patients with SSR-positive tumor have a better prognosis (Foekens *et al.*, 1989*b*). Tumors of these patients show a correlation between SSR and estrogen receptor expression and an inverse correlation between SSR expression and EGF receptor expression (Reubi and Torhorst, 1989; Reubi *et al.*, 1989). Papotti and co-workers reported a correlation between SSR expression and neuroendocrine differentiation in a series of 100 primary breast tumors (Papotti *et al.*, 1989*a*).

Alterations in oncogenes and tumor-suppressor genes are the fundamental changes that can lead to tumor formation. In breast cancer a number of genetic changes have been identified: loss of heterozygosity of the chromosome regions 1q, 3p, 11p, 13q14 (*RB* gene), 17p13, 18q and amplifications of the *int*-2, *myc* and *neu* oncogenes (van de Vijver *et al.*, 1988; Callahan, 1989; Devilee *et al.*, 1989; Varley *et al.*, 1989; Larsson *et al.*, 1990). At present it is not clear whether these genetic alterations are equally important in all types of breast cancer or whether a particular combination of events is present in a histologically and clinically distinct subgroup of tumors (Wong *et al.*, 1986; Harbour *et al.*, 1988). The finding of *neu* amplifications predominantly in the comedo type of ductal carcinoma *in situ* (van de Vijver *et al.*, 1988), and loss of heterozygosity of regions of chromosome 22q in lobular carcinoma (Larsson *et al.*, 1990) strongly support this latter possibility.

The aim of this study was to investigate whether breast tumors expressing SSR are a distinct subgroup at the genetic level. To this end a series of primary breast tumors was collected and tested for SSR expression and genetic alterations. We chose genetic markers that are frequently altered both in breast carcinomas and in neural or endocrine tumors such as neuroblastoma and small-cell lung carcinoma. These are alterations of the retinoblastoma tumor-suppressor gene (*RB*) and/or amplification of members of the *myc* family of oncogenes. Amplifications of the *neu* and *int*-2 oncogenes were also studied since these frequently occur in breast cancer.

Furthermore, clinical and pathological characteristics of the tumors were investigated. In addition, to test whether the reported correlation between SSR expression and neuroendocrine differentiation was present in this population, 43 tumors were also examined for neuroendocrine differentiation characteristics.

MATERIAL AND METHODS

Patient material

Eighty-seven breast tumors were obtained from the surgical departments of the Dijkzigt Academic Hospital and the Dr. Daniel den Hoed Cancer Center in Rotterdam between 1984 and 1990. Immediately after removal of the tumor, one part was taken for histological examination and the remainder was snap-frozen and stored in liquid nitrogen until use. Genetic studies were begun on 27 tumors from our archives, that were selected for the presence or absence of the somatostatin receptor. All tumors were histologically classified according to the criteria of the Azzopardi *et al.* (1982).

Patient and tumor characteristics were compared with SSR status of the tumors. Data were collected on age at onset, estrogen receptor status, lymph-node involvement, histology, stage and differentiation grade of the tumors. These data are summarized in Table I.

Somatostatin receptor autoradiography

Frozen tumor samples were shipped on dry ice to the Sandoz Research Institute in Bern (Switzerland), where the somatostatin receptors were measured by autoradiography on 10-μm cryostat sections. The size of the sections was over 100 mm^2. As a ligand, the iodinated stable analogue of somatostatin ^{125}I-[Tyr]^3-Octreotide was used. Incubation and washing conditions were as described (Reubi *et al.*, 1989). Non-specific binding was determined by adding unlabeled [Tyr]^{3}Octreotide. Results were scored on a semi-quantitative scale. A tumor was

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Received: April 18, 1992 and in revised form February 22, 1993.

TABLE I – CLINICAL AND PATHOLOGICAL DATA FROM 87 PRIMARY BREAST TUMORS

	ALL	SSR⁻	SSR⁺		
			Total	Homogeneous	Heterogeneous
Number	87[6]	29	58	41	17
Age[1]	57,5	60	56,2	56,9	54,4
ER[2]	45/64(70%)	14/21(67%)	31/43(72%)	20/28(71%)	11/15(73%)
Nodes[3]	31/73(42%)	12/26(46%)	19/47(40%)	14/33(42%)	5/14(36%)
Stage[4] pT1	30/81(37%)	8/29(28%)	21/52(40%)	17/37(46%)	4/15(28%)
pT2	38/81(46%)	16/29(55%)	22/52(42%)	15/37(40%)	7/15(47%)
pT3	6/81(7%)	1/29(3%)	5/52(10%)	3/37(8%)	2/15(13%)
pT4	8/81(10%)	4/29(14%)	4/52(8%)	2/37(5%)	1/17(7%)
Grade[5] I	15/84 (18%)	4/29(14%)	11/55(20%)	6/38(16%)	5/17(29%)
II	27/84(32%)	8/29(28%)	19/55(35%)	13/38(34%)	6/17(35%)
III	42/84(50%)	17/29(59%)	25/55(45%)	19/38(50%)	6/17(35%)

[1]Mean age in years.–[2]> 10 fmol/mg cytosol protein, measured by ligand binding assay or enzyme immunoassay (Foekens *et al.*, 1989*a*).–[3]Post-surgical regional lymph nodes present.–[4]pT post-surgical primary tumor stage according to TNM classification.–[5]I, highly differentiated; II, moderately differentiated; III, poorly differentiated.–[6]For specific subgroups, the numbers do not add up to 87 due to missing values.

counted as positive when specific binding was at least twice as high as the non-specific binding obtained by incubation in the presence of non-radiolabeled somatostatin analogue. Positive results were further classified as homogeneously positive when the whole section of tumor tissue stained positive. A tumor was heterogeneously positive when patches of positive cells were present in the tumor section.

Histology and immunohistochemistry

Sections of 5 μm were cut from formalin-fixed, paraffin-embedded tumor tissue. For histological examination the sections were stained with either hematoxylin and azophloxin or hematoxylin and eosin. To examine neuroendocrine differentiation, the original Grimelius silver impregnation method was used. Other neuroendocrine differentiation markers were examined using serial sections collected on poly-L-lysine treated slides and processed for immunohistochemistry. Antibodies against neuron-specific enolase (NSE) (DAKO, Copenhagen, Denmark), chromogranin A (Enzo, New York, NY) and chromogranin B (Dr. H. Winkler, Innsbruck, Austria) were used as described by Papotti *et al.* (1989*b*).

Southern hybridizations

Between 5 and 10 μg of DNA, isolated from frozen tumor samples and peripheral blood lymphocytes (as control), were digested with HindIII restriction enzyme, separated by electrophoresis on 0.8% agarose gels, and transferred to Hybond N⁺ nylon membranes (Amersham, Aylesbury, UK). The membranes were hybridized to ^{32}P-oligolabeled DNA probes. Hybridization and washing procedures were performed under standard conditions. After autoradiography on XAR films (Eastman Kodak, Rochester, NY) for 1 to 7 days, the membranes were stripped using 0.5% $NaDodSO_4$ (100°C, 15') and re-hybridized. Hybridization to a myoglobin (MB) DNA probe served as an internal control for the amount of DNA in each lane and the level of amplification. The following probes were used: *RB*, a 0.9 and a 3.8-kb EcoR1 cDNA fragment; *int*-2, a 1.0-kb BamH1-Kpn1 BK4 fragment; c-*myc*, a 1.6-kb Cla1-EcoR1 fragment; L-*myc*, a 1.8-kb Sma1-EcoR1 fragment; N-*myc*, a 1.0-kb EcoR1-BamH1 fragment; *neu*, a 1.6-kb cDNA fragment and *myoglobin*, a 0.6-kb BglII-EcoR1 per-made fragment.

RESULTS

Somatostatin receptor expression

Somatostatin receptor expression was assessed by autoradiography using a radiolabeled somatostatin analogue, [Tyr]^3-octreotide. In our series of 87 consecutive tumors, SSR expression was detected in 58 tumors (67%). In 41 of these, (47%) SSR expression was homogeneous and in 17 (20%) heterogeneous receptor expression was observed. The remaining 29 tumors (33%) showed no detectable SSR expression. An example of an autoradiogram showing heterogeneous receptor expression is given in Figure 1.

Neuroendocrine differentiation

Neuroendocrine differentiation was tested in 43 samples from 52 non-selected tumors used in the genetic studies, from which paraffin-embedded tumor tissue was available. The tumors were reviewed for neuroendocrine differentiation characteristics such as solid growth, lack of exocrine differentiation and monotonous cell morphology. In addition, sections were stained by the Grimelius procedure and consecutive sections were incubated with antisera against neuroendocrine differentiation markers, neuron-specific enolase (NSE) and chromogranin A and B. The results are listed in Table II.

NSE positivity was detected in 17 samples. In 4 tumor samples 3, 3, 5 and 20% of the tumor cells, respectively, stained positive with the Grimelius procedure. In one of the tumors positivity for chromogranin A was detected in 5% of the cells. In another tumor 5% of the tumor cells were positive for chromogranin B.

We arbitrarily scored a tumor as having a neuroendocrine differentiation when it was positive for at least 2 of these markers. Three tumors met this criterium: case 45 showed neuroendocrine morphological characteristics and was NSE positive, but negative for all other neuroendocrine markers; case 18 showed Grimelius and chromogranin A positivity in 3 and 5% of the cells, respectively; case 43 was NSE and Grimelius-positive in 50 and 20% of the cells. These 3 tumors also expressed the somatostatin receptor and are regarded as having a weak neuroendocrine differentiation.

Genetic alterations

Genetic alterations of the *RB* gene and amplification or alteration of the *neu*, *int*-2, c-*myc*, L-*myc* and N-*myc* oncogenes were investigated on Southern blots in 79 primary breast tumors. Thirty-six tumors expressed the SSR homogeneously, 11 expressed the receptor heterogeneously and in 32 tumors no somatostatin receptors could be detected. The tumors were not otherwise stratified. The results are shown in Table III. Deletion or rearrangement of the *RB* gene was detected in 10 tumors, of which 3 showed homogeneous SSR expression, 2 heterogeneous SSR expression and 5 were SSR-negative. *neu* amplifications were detected in 4 SSR-positive and 4 SSR-negative tumors. In 2 SSR-positive and one SSR-negative sample, *int*-2 amplification was observed. One of these samples

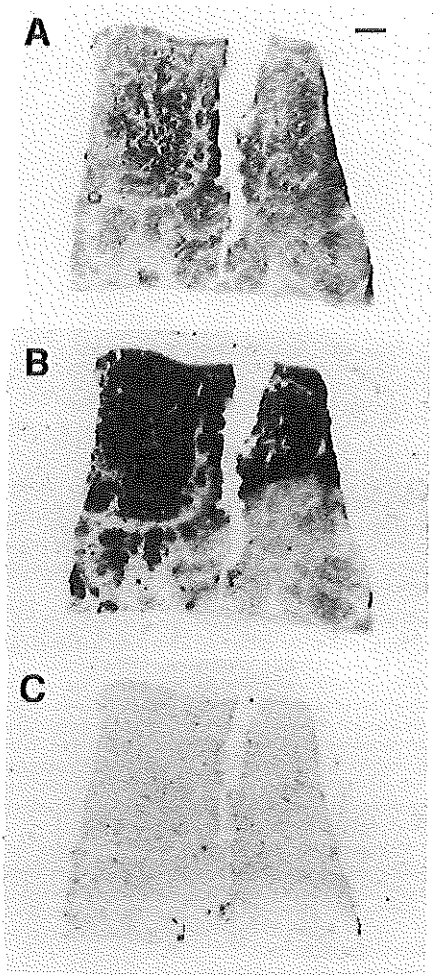

FIGURE 1 – Heterogeneous expression of somatostatin receptors. *(a)* Hematoxylin and eosin stain of a section of a primary breast carcinoma, showing tumor tissue throughout the section. *(b)* Autoradiogram showing total binding of radiolabeled somatostatin analogue ^{125}I-[Tyr]^3-Octreotide to a serial section of the same tumor. *(c)* Autoradiogram of the same tumor after incubation of labeled ^{125}I-[Tyr]^3-Octreotide in the presence of 10^{-6} M of unlabeled ^{125}I-[Tyr]^3-Octreotide, showing non-specific binding. Bar: 1 mm.

TABLE II – NEUROENDOCRINE MARKERS IN PRIMARY BREAST TUMORS[1]

Case	Histology[2]	NSE	Chrom.A	Chrom.B	Grimelius	SSR
1	–	+	—	—	—	–
3	–	+	—	—	—	+
6	–	+	—	—	—	+
15	–	–	—	5%	—	+
18	–	–	5%	—	3%	++
20	–	+	—	—	—	++
22	–	+	—	—	—	++
23	–	–	—	—	3%	–
25	–	+	—	—	—	+
26	–	+	—	—	—	+
27	–	+	—	—	—	+
28	–	++	—	?[3]	—	++
31	–	–	—	—	5%	–
37	–	+	—	—	—	–
39	–	+	—	—	—	++
43	–	+	—	—	20%	+
45	+	+	—	?[3]	—	+
49	–	+	—	—	—	++
50	–	+	—	—	—	++
51	–	+	—	—	—	+

[1]Only those tumors that were positive for one or more markers are listed.–[2]Presence of histological characteristics of neuroendocrine differentiation: solid growth, lack of exocrine differentiation and monotonous cell morphology.–[3]Non-specific result.

TABLE III – GENETIC ALTERATIONS IN 79 PRIMARY BREAST TUMORS IN RELATION TO SOMATOSTATIN RECEPTOR EXPRESSION

Gene alteration		SSR expression		
		Homogeneous	Heterogeneous	Negative
RB	Yes	3(8%)	2(18%)	5(16%)
	No	33	9	27
Neu	Yes	4(11%)	0	4(12%)
	No	32	11	28
int-2	Yes	2(5%)	0	1(3%)
	No	34	11	31
c-*myc*[1]	Yes	1(3%)	1(12%)	0
	No	29	7	30
L-*myc*[2]	Yes	1(3%)[3]	0	0
	No	30	10	30
N-*myc*	Yes	0	0	0
	No	36	11	32

[1]68 tumors were tested for c-*myc* amplification and [2]71 tumors for L-*myc* alterations.–[3]rearrangement.

showed co-amplification of the *neu* and *int*-2 oncogenes, and one had both a deleted *RB* gene and *int*-2 amplification. C-*myc* amplification was observed in 1 SSR-positive tumor. Rearrangement of the L-*myc* oncogene was detected in 1 SSR-positive tumor. No alterations of the N-*myc* gene were detected. Compared to the hybridization signal of the myoglobin gene, 5- to 15-fold amplifications were observed. Examples of such rearrangements and amplifications on Southern blotting are shown in Figure 2. In Figure 2*a*, lanes 1–8 contain HindIII-digested tumor DNAs and lane N HindIII-digested control DNA from normal peripheral lymphocytes. This Southern blot was hybridized to a 3′ *RB* cDNA probe. Two tumor DNAs show a clear rearrangement of the *RB* gene. The tumor in lane 5 shows an extra equimolar 3.2-kb fragment, and in lane 3 the top 2 hybridizing fragments of 9.8 and 7.8 kb are less intense than the other hybridizing fragments in this tumor, indicating a partial deletion of the *RB* gene. Figure 2*b* shows HindIII-digested tumor DNAs, hybridized with probes for the *neu* and c-*myc* oncogenes. The hybridization pattern of the myoglobin gene (*MB*) is included as a control for the amount of DNA. A *neu* amplification is visible in lane d where, compared to the MB signal, the *neu*-signal is 5 times stronger.

DISCUSSION

In our series of 87 primary breast tumors, we detected SSR expression in 67% of the tumors. The incidence of SSR expression was much higher than that reported in the literature to date. However, concurrently with this study, we screened a partly overlapping group of 50 patients for SSR expression using an *in vivo* SSR-scanning technique prior to

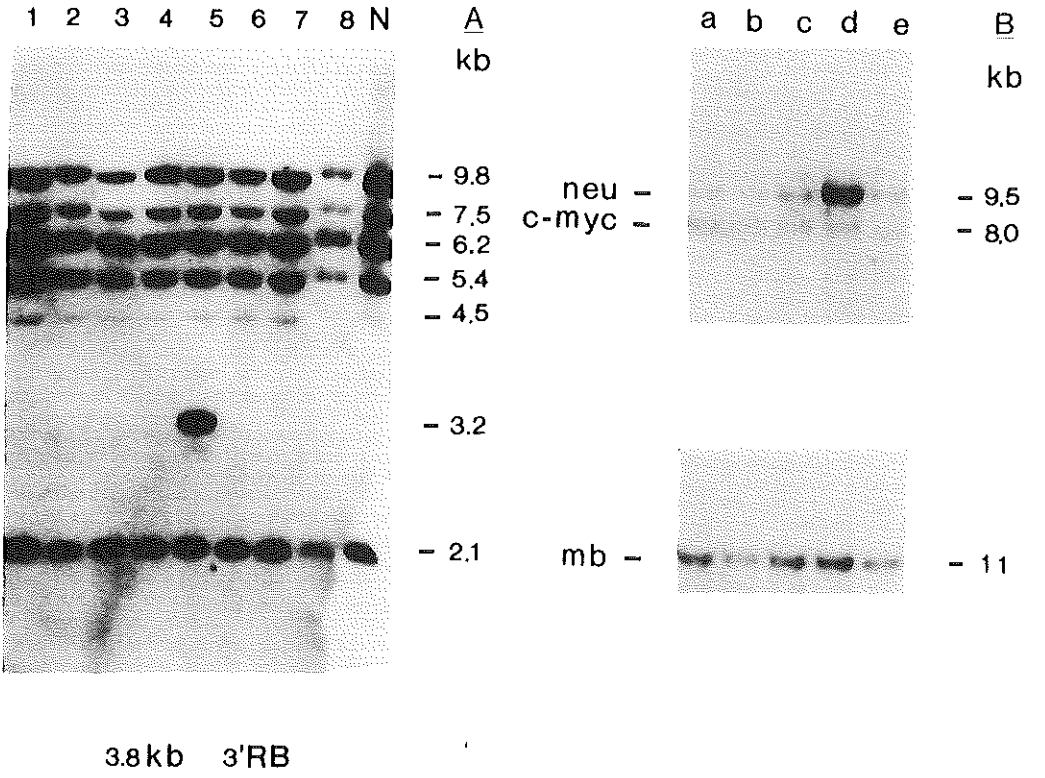

FIGURE 2 – Genetic alterations in primary breast tumors. *(a)* Autoradiogram of a Southern blot containing HindIII-digested DNA from 8 different primary breast tumors in lanes 1 to 8 and from lymphocyte control DNA in lane N. The blot was hybridized to a 3.8-kb *RB* cDNA probe. *(b)* Autoradiogram of a Southern blot containing HindIII-digested DNA from 5 different primary breast tumors, after hybridization to *neu* and *c-myc* gene probes. The DNAs were also hybridized with a myoglobin gene (*MB*) probe as a control for the amount of DNA applied to each lane.

operation. In this group of patients, 75% of the tumors were positive for SSR expression (data not shown). Other studies on SSR expression in breast cancer report an incidence of 10 to 47%. Fekete *et al.* (1989) detected binding of somatostatin in 36% of 500 breast cancer biopsy homogenates. Using autoradiography, Reubi and Torhorst (1989) and Papotti *et al.* (1989*a*) detected specific somatostatin analog binding in 17%–20% of primary breast tumors. However, in a more recent study Reubi *et al.* (1989) detected somatostatin analog binding in 46% of primary breast tumors. In that study much larger tumor sections (180 ± 8 mm²) were examined for SSR expression than previously. Over 50% of the SSR-positive tumors showed heterogeneous SSR expression, which had not been detected previously and which may explain the 2-fold difference in incidence upon comparison with earlier studies.

In the present study we also used large tumor sections (> 100 mm²) for autoradiography. However, in this series only 17 of the 58 SSR-positive tumors showed heterogeneous SSR expression. Thus, a lower detection limit as a result of the use o larger tissue sections and subsequent detection of more heterogeneous SSR-positive tumors cannot fully explain the high incidence of SSR-positive tumors in this series of breast tumors.

Compared with other reports (Foekens *et al.*, 1989*b*; Papotti *et al.*, 1989*a*; Reubi and Torhorst, 1989; Reubi *et al.*, 1989) our tumor series showed a similar distribution of age at onset and estrogen receptor expression. Moreover, no difference was observed between the SSR-positive and SSR-negative tumors. However, our tumor population contained a high percentage of T1/T2 stage tumors (83%). This high percentage was found in both the SSR-positive and the SSR-negative subgroups, but the frequency of T1 tumors was higher in the SSR-positive group. In only one other study on somatostatin receptor expression (Reubi *et al.*, 1987), has tumor size been reported. In that report T1 and T2 stages made up 59% (16/27) of the tumors and 3/27 of these tumors were SSR-positive.

A trend towards smaller tumor size in the Dutch breast tumor population was also observed in a large study by Coebergh *et al.* (1990), who reported a 2-fold rise in incidence of pT1 tumors (28 to 42%) and a 50% reduction of pT3/pT4 tumors in the years 1970–1986. The authors suggested that this shift towards smaller tumors could be due to earlier detection as a result of population screening programs. Another factor contributing to the low percentage of later-stage tumors in our series was the policy of the Surgery Department of the Dijkzigt Academic Hospital not to operate on patients with a clinically

assessed T4 tumor. These T4 tumors were therefore seldom available for study. This bias in our set of tumors should be borne in mind when comparing the results of the present study with those of other studies.

Therefore, a possible explanation for the high incidence of somatostatin receptor expression reported in this study could be the combination of a high percentage of pT1/pT2 tumors and the large size of the tumor sections (not of the tumors themselves) used for SSR-autoradiography, which allowed the detection of heterogeneous receptor expression.

Small tumor size has been correlated to a favorable prognosis and recently also to a lower differentiation grade (*i.e.* better tumor differentiation) (Tubiana and Koscielny, 1991). Our data show a tendency towards a lower differentiation grade in SSR-positive tumors. This is concordant with clinical data assigning a favorable prognosis to patients with SSR positive-tumor.

Since SSR expression has been correlated to neuroendocrine differentiation in other tumor types, such as lung cancer, we tested 43 tumors for expression of other markers of neuroendocrine differentiation. Seven percent (3/43) of these tumors showed neuroendocrine differentiation, which is within the range of 5–10% reported by others (Papotti *et al.*, 1989*a,b*). Since we found a normal low frequency of neuroendocrine differentiation, but a high incidence of SSR expression in the same series, we conclude that SSR-expressing breast tumors and those showing neuroendocrine differentiation are in this series overlapping, but independent, subgroups.

In a selected group of 79 tumors we investigated whether any of the genetic alterations that are frequently observed in breast tumors were specific for the SSR-positive or SSR-negative subgroups. The Southern-blot method, used to detect changes in the *RB* gene and the *neu, int-2* and *myc* oncogenes, does not permit identification of all possible alterations in these genes, but is useful for detecting subgroup specificity of these alterations. Our results indicate that no significant correlations exist between the presence or absence of the SSR and loss of the *RB* tumor-suppressor gene. Similarly, none of the investigated oncogene amplifications or rearrangements are specific for the subgroup of SSR-positive or SSR-negative breast tumors. The *RB* and *myc* genes were chosen because they were reported to be altered in both breast cancer and neuroendocrine tumors. Our observation that these alterations also occur in SSR-negative tumors lacking neuroendocrine markers suggests that these genetic changes are not restricted to breast tumors with neuroendocrine differentiation.

Nesland *et al.* (1991*a,b*) reported that *neu* amplification and neuroendocrine differentiation are mutually exclusive in breast tumors. In our series we detected 3 breast tumors with weak neuroendocrine differentiation. None of these tumors contained an amplified *neu* oncogene. This observation is in line with that of Nesland *et al.* (1991*b*), although the numbers are small.

No other reports have been published on the relation between genetic alterations and expression of the somatostatin receptor in breast tumors. Some data are, however, available on the relationship between amplification of the *neu* oncogene and expression of the EGF receptor (EGFR). A correlation between *neu* over-expression and EGFR expression was reported by Marx *et al.* (1990). This observation was in contrast to the results of others who did not find such a correlation (Zeilinger *et al.*, 1989; Moe *et al.*, 1991). In the present study SSR content of the tumors was assessed. However, we have reported earlier that SSR and EGFR expression are mutually exclusive in breast cancer (Reubi *et al.*, 1989). This implies that the observed lack of correlation between SSR expression and *neu* amplification, as reported in the present study, and the data on the relation between *neu* amplification and EFGR expression, accord with each other.

It is worth while to test other genetic markers in the subgroup of SSR-positive breast tumors, because the somatostatin-receptor-expressing breast tumors are a clinically relevant subgroup, and also because a high percentage of early-stage tumors in this subgroup gives an opportunity to study those genetic alterations that are involved in the initial steps of tumor formation.

ACKNOWLEDGEMENTS

We are grateful to Drs. G. Bussolati and M. Papotti (Dept. of Biomedical Sciences and Human Oncology, University of Turin) for their contribution to the neuroendocrine part of the study and for critically reading the manuscript. We thank Dr. D. Bootsma for support and advice. This study was supported by Sandoz Research Foundation in The Netherlands (A.H.B.), the Royal Dutch Academy of Science (A.d.K.) and the Dutch Cancer Society (K.K.S.).

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III.2: SOMATOSTATIN-RECEPTOR SCINTIGRAPHY IN PRIMARY BREAST CANCER.

Lancet, 343: 640-643, 1994.

Reprinted from THE LANCET, *March 12 1994, pp. 640–643*

Somatostatin-receptor scintigraphy in primary breast cancer

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Summary

Somatostatin-receptor (SS-R) scintigraphy successfully shows primary cancers and distant metastases in most patients with carcinoids, islet cells tumours, and paragangliomas. Previous in-vitro studies indicated that somatostatin receptors are present in human breast cancers.

We report positive scintigraphy with [^{111}In-DTPA-D-Phe^1]-octreotide in 39 of 52 primary breast cancers (75%). Parallel in-vitro autoradiography with [^{125}I-Tyr^3]-octreotide of 30 of these showed a corresponding somatostatin-receptor status in 28. Significantly more invasive ductal cancers could be shown than invasive lobular carcinomas (85% *vs* 56%; $p<0.05$). Also the number of T_2 cancers which were shown was higher than T_1 (86% *vs* 61%; $p<0.05$). Imaging of the axillae showed non-palpable cancer-containing lymphnodes in 4 of 13 patients with subsequently histologically-proven metastases. In the follow-up after a mean of 2·5 yr, SS-R scintigraphy in 28 of the 37 patients with an originally SS-R-positive cancer, was positive in the 2 patients with clinically-recognised metastases, as well as in 6 of the remaining 26 patients who were symptom-free. Raised carcinoembryonic antigent (CEA) and CA 15-3 values were observed in only 2 and 1, respectively, of these patients.

Most primary breast cancers can be shown by SS-R scintigraphy, especially invasive ductal cancers. This technique may be of value in selecting patients for clinical trials with somatostatin analogues or other medical treatments. Furthermore, SS-R scintigraphy is more sensitive than measurements of the usual serum cancer markers for detecting recurrences of SS-R-positive breast cancer.

Lancet 1994; **343:** 640–43

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Introduction

Somatostatin receptors (SS-R) have been found in neuroendocrine tumours (carcinoids, islet cell tumours, paragangliomas) as well as in meningiomas.[1-5] Studies also show SS-Rs in some primary breast cancers,[6-9] probably of neuroendocrine origin.[8] Antiproliferative effects of somatostatin analogues have been reported on the growth of experimental cancers, including breast-cancer cell lines and explants.[10,11] We recently introduced a new technique in which SS-R-positive cancers could be shown in vivo, after giving radionuclide-labelled somatostatin analogue followed by gamma-camera scintigraphy.[12-16] In the present study we investigated this technique in primary cancers and metastates of 50 patients with breast cancer, and compared its value in the detection of recurrent disease with the serum markers CEA and CA 15-3, which are commonly used for this purpose.

Patients and methods

We studied 50 patients with 52 primary breast cancers (mean age 61; range, 38–93). After clinical examination and mammography, cancers were cytologically confirmed to be primary breast cancer. When patients gave informed consent to this study, blood samples were taken for measurements of cancer markers and SS-R scintigraphy was done as an outpatient. After scanning, all patients had an operation within 14 days, except for one, who had chemotherapy due to a T_4 breast cancer. Preoperative physical examination and chest X-ray showed no evidence of metastatic disease, nor were there clinical signs of local spread. 27 patients were treated by removal of the lump and axillary dissection, 22 with modified radical mastectomy (2 patients had a bilateral modified radical mastectomy). The Scarff, Bloom, and Richardson grade (SBR) was assessed by one pathologist (R v P) in all patients. The presence of somatostatin receptors in 30 of these cancers was measured by in-vitro autoradiography (J C R) on cryostat sections of cancer tissue, as has been described previously.[1,9]

The somatostatin analogue [DTPA-D-Phe^1]-octreotide (Mallinckrodt Medical BV, Petten, Netherlands) was labelled with ultra-pure ^{111}Indium.[16] Doses ranged from 200 MBq to 272 MBq [^{111}In-DTPA-D-Phe^1]-octreotide. Planar images were obtained with a large field-of-view gamma camera (Counterbalance 3700, Siemens Gammasonics, Erlangen, Germany) equipped with a 190-KeV parallel-hole collimator. Generally, the field of view covered the chest and the upper part of the abdomen. 24 hours after injection of [^{111}In-DTPA-D-Phe^1]-octreotide, chest images were obtained anteriorly and posteriorly, with additional images of the axillary region with arms elevated. 500 Kcts were collected per image with a maximum counting time of 15 min. A simple high/moderate, low/negative system was used to define the accumulation of radioactivity by the tumours as seen during scanning, carried out by E P K and H Y O, who were not informed where the cancer was.

Serum samples were stored at −20°C before assay. Serum CEA was determined by enzyme-linked immunoassay (ELISA) kits (Boehringer, Mannheim, Germany) (normal upper limit normal concentration 10 ng/mL). Serum CA-15·3 was determined by ELISA (Centeroor, Leiden, Netherlands).

Results

39 of the 52 tumours were seen on [^{111}In-DTPA-D-Phe^1]-octreotide scintigraphy. The intensity of scintigraphy varied considerably. In figure 1, the SS-R scan is shown of a 70-year-old with bilateral cancer and autoradiographic results obtained on a section of cancer removed from this patient, showing specific binding of [^{125}I-Tyr^3] octreotide. In 30 of these 52 cancers, autoradiographic studies of the surgically-removed tissue for the presence of SS-R could be done in parallel to the scintigraphy with [^{111}In-DTPA-D-Phe^1]-octreotide. SS-R's were present in 23 cancers; comparison between in-vivo scintigraphy and in-vitro autoradiography showed that receptors were found in both instances in 22, and absent on both investigatons in 6. A discrepancy between the in-vivo and in-vitro results was observed in 2. In one cancer, a non-homogeneously sparse distribution of SS-R's was found at autoradiography, while the tumour was not seen in vivo; in the other, low radioactivity was seen on the scintigram of the breast containing a SS-R-negative cancer according to autoradiography. Two types of SS-R distribution were recognised at autoradiography: in 16 the receptors were homogeneously and often densely distributed over the cancer tissue, while they were found to be non-homogeneously scattered in 7, all non-invasive ductal cancers.

Cancers showing a dense distribution of SS-R's in vitro were most clearly seen in vivo. Figure 2 shows the scan of a 56-year-old with a T_2 ductal carcinoma with a large non-invasive component. Autoradiography with [^{125}I-Tyr^3]-octreotide shows only specific binding of somatostatin throughout the non-invasive component of the tumour. Figure 3 shows the scintigram of a 39-year-old patient with a T_2 invasive ductal carcinoma of the left breast. Physical examination did not reveal palpable lymph nodes in the axilla. The scan, however, showed radioactivity in the axilla, and histology confirmed axillary lymph-node metastases. Of the subsequent 13 consecutive patients with histologically-proven non-palpable axillary

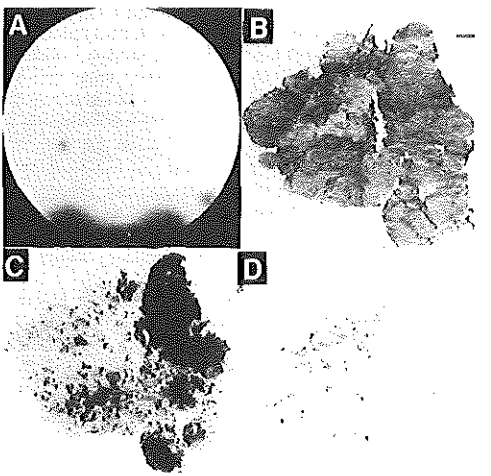

Figure 1: A: [^{111}In-DTPA-D-Phe^1]-octreotide scintigraphy showing bilateral breast cancers. B: Haematoxylin-eosin stained section. C: Autoradiogram showing total binding of [^{125}I-Tyr^3]-octreotide. D: Autoradiogram showing non-specific binding of [^{125}I-Tyr^3]-octreotide (in presence of 10^{-6} M Tyr^3-octreotide).

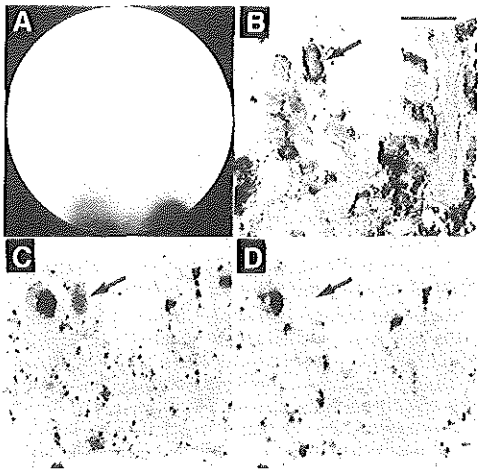

Figure 2: A: [^{111}In-DTPA-D-Phe^1]-octreotide scintigraphy of a 56-year-old patient showing cancer faintly in the right breast. B: Haematoxylin-eosin stained sections containing non-invasive tumour tissue, ductal carcinoma-in-situ (DCIS). C: Autoradiogram showing total binding of [^{125}I-Tyr^3]-octreotide. Only one limited region containing DCIS is strongly labelled. D: Autoradiogram showing non-specific binding of [^{125}I-Tyr^3]-octreotide (in presence of 10^{-6} M Tyr^3-octreotide).

lymph node metastases and a positive SS-R scan of the primary cancer, lymph-node metastases were seen in 4. None of the patients with a negative scan of the primary cancer showed abnormal radioactivity in the axillae or elsewhere in the body.

There was no correlation between [^{111}-In-DTPA-D-Phe^1]-octreotide positivity of the cancers in vivo and age (<60 yr 15/18 [83%]; >,60 yr 24/34 [71%]). 85% of the ductal cancers could be seen and 56% of the lobular cancers ($p < 0.05$). Also, significantly more T_2 cancers were seen than T_1 cancers ($p < 0.05$; table 1). 37 patients with 39 SS-R-positive primary breast cancers were selected to have repeat SS-R scintigraphy on average 2·5 yr after initial treatment (23–36 months). Of these patients, 2 had died (one due to metastatic breast cancer), 3 were bedridden at home (2 because of metastatic breast cancer), 2 had been discharged from follow-up, and 2 refused. Of the remaining 28 with an originally SS-R-positive primary breast cancer, 2 had already had repeat scintigraphy because of suspected metastases. In one of these, SS-R-positive metastases had been shown in the liver, lung, and cervical spine; and in the other, both axillary and mediastinal lymph-node metastases

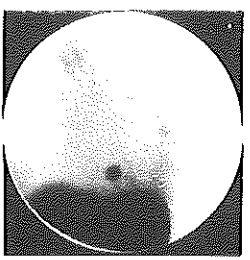

Figure 3: [^{111}In-DTPA-D-Phe^1]-octreotide scintigraphy of a 39-year-old patient showing breast cancer on the left side and axillary-node metastases.

	No of cancers	Positive SS-R scan (%)
Cancer size		
T_1	23	14 (61%)
T_2	28	24 (86%)*
T_4	1	
Histological stage		
T_1N_0	15	9 (60%)
T_2N_0	14	12 (86%)
T_1N_1	8	5 (62%)
T_2N_1	9	8 (89%)
T_2N_2	5	4 (80%)
T_4N_2	1	1

* < 0.05 vs T_1.

Table 1: [^{111}In-DTPA-D-Phe^1]-octreotide uptake in primary breast cancer related to cancer size and stage

were seen on the side of the original breast cancer. 26 patients were symptom-free; physical examination and scintigraphy showed lesions suspected to be metastases which were confirmed by biopsy, bone scan, ultrasound, or computerised tomographic scan: bone (4); liver (1); pulmonary (2); pleural (1); and in axillary (2), infraclavicular (1), and mediastinal (1) lymph nodes. In one patient, treated by cancer removal and axillary dissection, a local recurrence was seen at SS-R scintigraphy (figure 4).

14 patients with an originally SS-R-positive primary cancer, had a normal somatostatin scan on follow-up. Slight scattered radioactivity distributed over one lung was seen in 6 patients after radiotherapy following by cancer removal and axillary dissection. The 17 with an originally SS-R-negative scan were not rescanned; none have died and all are symptom-free.

Serum CA 15-3 and CEA were normal at first presentation in 35 and 37 patients respectively, with a SS-R-positive primary cancer and none of these cancer markers was raised in patients with primary SS-R-negative tumours, of which the SS-R status is based on SS-R scintigraphy. At follow-up, only one of the symptomatic patients had raised CA 15-3 and CEA, and also 1 of the asymptomatic patients had increased CA 15-3. All patients

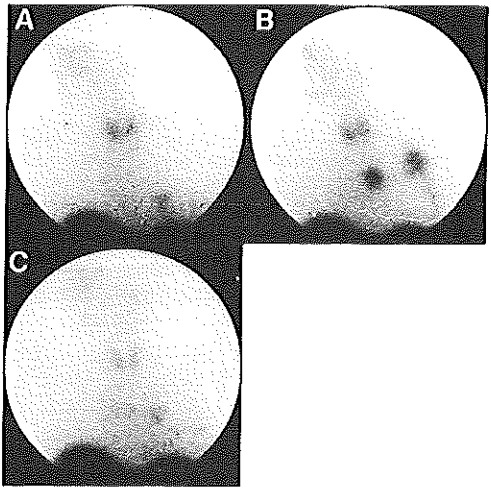

Figure 4: **A: 58-year-old patient with primary breast cancer of the left breast. B: 17 months later, mediastinal and axillary lymphnode metastases were shown on the left side. Treatment with chemotherapy followed. C: Thirteen months later there was complete disappearance of the axillary cancer and a partial response of the mediastinal cancer infiltration**

	CA 15-3 (U/mL)		CEA (ng/mL)	
	Admission	Follow-up	Admission	Follow-up
Symptomatic (n=2)	17	23	2	2
	17	331	1	30
Asymptomatic (n=8)	11	14	1	1
	42	92	1	2
	11	11	1	1
	22	20	1	1
	22	22	8	8
	25	21	2	2

Symptomatic: patients with clinically-overt metastatic disease.
Asymptomatic: patients without clinically-overt metastatic disease.

Table 2: **CA 15-3 and CEA in patients with SS-R-positive primary breast cancers and proven recurrent disease according to SS-R scintigraphy at follow up of 30 months**

with a normal SS-R scan at follow-up had serum cancer-marker values in the normal range except for 1 who had a slightly elevated CA 15-3 as was the case at first presentation (table 2).

Discussion

SS-R imaging has been shown to be successful in the identification of primary as well as metastatic cancer sites of a variety of neuroendocrine tumours.[12-16] Validation of the technique was by in-vitro demonstration of high-affinity binding sites for somatostatin in those cancers, which had been shown in vivo.[14] A positive SS-R scan closely predicted a beneficial effect of octreotide treatment on hormonal hypersecretion by these tumours.

Some breast cancers contain SS-Rs measured in vitro. In a group of 158 small breast-cancer samples (mean section surface 14 mm^2) 34 (21%) were SS-R positive, while in a group of 72 larger cancer samples (mean section surface 180 mm^2) 33 tumours (46%) were SS-R positive.[6] A subpopulation of SS-R-positive breast cancers is probably of neuroendocrine origin.[8,17] The percentage of SS-R-positive cases varies not only with the specificity and sensitivity of the neuroendocrine markers used, but depended also on the number of tissue slices per cancer investigated microscopically.[8] We showed 39 of 52 primary breast cancers (75%) to be somatostatin-receptor positive by scintigraphy. There was a close correlation between the in vivo [^{111}In-DTPA-D-Phe^1]-octreotide scan and subsequent in vitro autoradiography with ^{123}I-Tyr^3-octreotide. There was variability in radioactivity at SS-R scintigraphy; higher density in vivo correlated mostly with homogeneous and dense distribution of SS-Rs at autoradiography, while lower density of radioactivity over the cancer area in vivo corresponded with a non-homogeneous and sparse distribution of these receptors in vitro. Interestingly, low density of receptors seemed to be due to a non-invasive cancer component, mainly ductal carcinoma-in-situ.

The high incidence of SS-Rs in these 52 cancers, as observed by scintigraphy might be due to several causes. Firstly, in-vivo SS-R demonstration of breast cancer may be more sensitive than in-vitro autoradiography, as scintigraphy shows the presence of receptors in the whole cancer. In accordance with this, statistically significantly more T_2 than T_1 cancers were seen in vivo. Secondly, our patients might represent a selected group in comparison with those from other countries, as the incidence of ductal cancers amongst patients with newly-diagnosed breast cancers has increased over the last years in the Netherlands following the introduction of routine and repeated screening of the population.[19] Little is known about the

biological behaviour of SS-R-positive breast cancer, although a retrospective study of 110 patients[20] suggested that the presence of SS-R might predict a longer disease-free survival. Also, in-vitro studies of more than 300 breast-cancer samples showed an inverse relationship between somatostatin and EGF-receptor expression.[6,9] These observations suggest that patients with SS-R-positive cancers might have a relatively good prognosis. This, however, is not substantiated by our observations. After a mean follow-up of 2·5 yr we found that of 37 SS-R-positive patients 5 had extensive metastases, and also 6 of 26 symptom-free patients had metastases initially shown by SS-R-receptor scintigraphy.

CA 15-3 and CEA are the most commonly-used cancer markers to monitor patients with recurrent breast cancer. Both were raised in only 5–20% of women with primary breast cancer, but elevations between 61% and 84% have been recorded for women with extensive metastatic disease. CA 15-3 seems to be related to the extent of the metastases, the number of metastatic sites and survival, whereas CEA is only correlated with the extent of disease.[21,22] We show a higher sensitivity of SS-R scintigraphy compared with these cancer markers in detecting the development of recurrence in patients with SS-R-positive primary breast cancer. SS-R scintigraphy showed recurrent disseminated breast cancer in 6 (only 1 of whom had symptoms of recurrence) out of 28 patients with SS-R-positive primary cancers; all 6 patients had normal CA 15-3 and CEA. Another 3 out of these 28 patients had abnormal serum cancer markers, 2 of whom had an abnormal SS-R scintigram. The 3rd patient, who only showed marginally elevated CA 15-3 both at first presentation and follow-up, is clinically in remission 3·2 yr after the operation. Peptide-receptor demonstration of primary breast cancer with a radionuclide-labelled somatostatin analogue is successful in 75% of cases.

At primary diagnosis, the scintigraphic technique seems of minor value in the detection of axillary lymph node metastases; detection of these may be improved with guided surgery using a hand-held radio-nuclide detecting probe after administration of a radionuclide-labelled somatostatin analogue. The results so far also suggest that distant metastases of the primary somatostatin-receptor breast cancers continue to express such receptors at a mean of 2·5 yr after initial treatment. Scintigraphy showed the unexpected presence of metastases in nearly 25% of symptom-free initially SS-R-positive breast cancer patients, who had normal CA 15-3 and CEA serum values. The clinical relevance of detecting recurrent disease in an early stage is not known.

In-vitro studies with breast-cancer cell lines indicate a direct receptor-mediated inhibitory effect of somatostatin analogues on cell proliferation.[10,23-25] No prospective, controlled studies on the use of somatostatin analogue in patients with breast cancer have yet been published. As SS-R scintigraphy is effective in the early detection of SS-R-positive breast cancer recurrence, it may have future use in the selection of patients who can be treated with somatostatin analogues or radiotherapy with an α-emitting or β-emitting radionuclide coupled to a somatostatin analogue.

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III.3: ADDENDUM TO CHAPTERS III.1 AND III.2

In chapters III.1 and III.2 the expression of the somatostatin receptor in human breast cancer was investigated *in vitro* and *in vivo*. The consecutively collected tumours investigated in chapter III.1 partly derive from the patients under investigation in chapter III.2. Due to a difference in the period that the tumours were collected, the overlap is not complete. However, the patient and tumour characteristics in both studies are comparable. Also, the high incidences of somatostatin receptor expression *in vitro* and *in vivo* correspond well. Interestingly, the results of both studies conflict with respect to the significance of somatostatin receptor expression on the patient's prognosis. In chapter III.1 we suggest SSTR expression might correlate with a favourable prognosis. This was based on the correlation of SSTR expression with smaller tumour size. Also, earlier retrospective investigations had reported a favourable prognosis in SSTR-positive breast cancer. In contrast to these data are the first follow up data of the *in vivo* study (chapter III.2) that suggest more aggressive disease: Out of the 37 patients with a SSTR-positive tumour at the time of the first scanning, five patients (13%) developed clinically assessed recurrent disease during the 2.5 years of follow-up, whereas none of the 17 patients with an initially SSTR-negative tumour showed clinical evidence for recurrence of disease. In addition, six symptom-free patients (16%) with a positive first scan were again positive on the second scan. The significance of this scan result may only be assessed after longer follow-up. Neither the patients with clinically recurred disease nor those with a second positive scan without other signs of disease recurrence were characterized by any specific patient or tumour characteristic. Genetic data were available from two patients only, not enough to reach a conclusion about possible common genetic characteristics. It appears that although SSTR positive tumours show a tendency towards smaller tumour size, this by no means implicates a better prognosis for the patient.

The explanation might be that recurrent disease can be detected at an earlier stage in SSTR-positive tumours by the sensitive [^{111}In-DTPA-D-Phe^{1}]-Octreotide scintigraphy method. However, before any conclusions can be reached, prospective survival studies are necessary.

III.4: CHROMOSOMAL LOCALIZATION OF TWO HUMAN SOMATOSTATIN RECEPTORS (SSTR1 AND SSTR2) AND ANALYSIS OF ALLELIC IMBALANCE IN PRIMARY HUMAN BREAST CARCINOMAS.

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Abstract

Somatostatin is an important neuro-endocrine regulator. Receptors for somatostatin are present in most neuronal and neuro-endocrine tissues and in a variety of tumours. Recently, five somatostatin receptor genes (*SSTR*) have been cloned. Binding of somatostatin to its receptor mainly causes inhibitory effects. Also an anti-proliferative effect has been observed in most somatostatin receptor positive tumours and cell lines, and as such somatostatin or somatostatin receptor genes may act as tumour suppressor genes.

A high percentage of primary breast cancers express somatostatin receptors and the highest incidence is observed in the smaller, stage pT1 and pT2 tumour samples. In 30% of the somatostatin receptor expressing tumours, a heterogeneous pattern of expression was seen. Whether this heterogeneity is the result of loss of SSTR expression, and if the absence of SSTR expression correlates with specific chromosome loss, is at present unknown. Using fluorescence *in-situ* hybridization (FISH) we have mapped the *SSTR1-4* genes on chromosome bands 14q13, 17q23-24, 22q13 and 20p11, respectively.

FISH of *SSTR1* and *SSTR2* probes on metaphases from the somatostatin receptor positive cell lines MCF-7 and ZR75-1 showed that in both cell lines more *SSTR2* gene copies were present than could be expected from the DNA ploidy of the cells. The number of chromosomal loci with *SSTR1* sequences was according to the DNA ploidy of the cells.

Allelic imbalance at the *SSTR1* and *SSTR2* loci was assessed in 96 primary breast tumour/normal tissue pairs, using polymorphic CA repeats in the vicinity of the SSTR genes. In approximately 50% of the tumours loss or gain of one of the allelic copies from either *SSTR* gene was observed. These results suggest that the somatostatin receptor genes could play a role in the genetic changes that eventually lead to the malignant transformation of human breast tissue.

Introduction

Somatostatin is a tetradecapeptide that acts as a neurotransmitter in the central nervous system, and has inhibitory effect on endocrine and exocrine secretory processes in several organs. Interestingly it also inhibits cell proliferation in some types of cancer[1]. This growth inhibitory effect of somatostatin has also been shown in breast cancer cells[2] and cell lines, like the *SSTR* expressing, MCF-7, T47D and ZR-75-1 breast cancer cell lines[3-6]. This effect is mediated by binding of somatostatin to the somatostatin receptor. The recent cloning of five *SSTR* genes confirmed the presence of a family of receptors, and showed that they belong to the class of seven transmembrane domains G-coupled receptors[7-12]. Since *SSTR*s are involved in the negative regulation of cell growth, they are candidate tumour suppressor genes. Loss of one or both alleles can give the tumour cell a growth advantage. For the retinoblastoma tumour suppressor gene it has been observed that the first allele is often inactivated by a small mutation within the gene, while the inactivation of the second allele occurs by a less subtle event, like loss of a part or of a whole chromosome arm[13], resulting in an allelic imbalance in the tumour DNA. A method to investigate the involvement of tumour suppressor genes in carcinogenesis is to assess allelic imbalance of these genes with polymorphic DNA probes. The constitutional genotype of cancer patients is then compared with the genotype of their tumours. In breast cancer allelic imbalance in over 20% of primary tumours has been reported for chromosomes 1, 3, 4, 6, 13, 16, 17, 18 and 22[14-16]. In other chromosome arms like 14q allelic imbalance has been observed in less than 20% of tumours[17]. Forty to 70 percent of the human primary breast tumours express somatostatin receptors. Interestingly, a heterogeneous pattern of somatostatin receptor expression has been observed in 30% of SSTR-positive primary breast tumours: some areas of tumour sections appear positively stained with radiolabeled ligand and others are negative[18]. At present it is not known if this is due to loss of SSTR expression in parts of the tumour and if specific chromosomal losses are related to this loss of expression. To investigate this possibility fluorescence *in situ* hybridization (FISH) experiments using SSTR1, SSTR2, SSTR3 and SSTR4

gene sequences were used to assess the chromosomal localization of these genes in normal human metaphases. The number of chromosomal loci with SSTR1 or SSTR2 sequences was investigated in two SSTR positive breast cancer cell lines: ZR75-1 and MCF-7, and compared with the DNA ploidy of the cells. Furthermore, in 93 primary breast tumour/ normal tissue pairs the integrity and allelic imbalance of the SSTR1 and SSTR2 loci were studied using CA repeats in the vicinity of these genes.

Materials and methods

FISH

Probes: Phages containing coding sequences of four SSTR genes were used as probes. SSTR1 and SSTR2 probes were isolated from a genomic EMBL library (Clonetech). The identity of the phages was confirmed by partial sequencing of the coding sequences with a T7 sequencing kit (Pharmacia). Genomic phages containing SSTR3 (ph SSTR2-15) and SSTR4 (hGL-5-1) were a gift from Dr. GI Bell and Dr. JF Bruno, respectively.

Method: Probes were labeled by nick-translation using Bio-16-dUTP (Boehringer Mannheim). After denaturation of probes and target metaphases, hybridization was carried out overnight according to our standard protocol[19]. Hybridization signals were detected with FITC-conjugated avidin (Vector Laboratories, Burlingam, CA). Chromosomes were identified by staining with 4,6-diamino-2-phenylindole dihydrochloride (DAPI).

SSTR localization was studied on normal human metaphases, prepared from PHA-stimulated peripheral blood lymphocyte cultures as described before[20]. For each probe at least 10-20 metaphases were scored. Metaphases of the two SSTR expressing breast cancer cell lines ZR75-1 (ATCC CRL1500) and MCF-7 (ATCC HTB-22) were also used.

CA-repeat detection: Allelic imbalance at the SSTR1 and SSTR2 loci was

investigated using the polymerase chain reaction (pcr) to detect CA repeat polymorphisms located in the SSTR1 and SSTR2 locus, as described[21]. The dinucleotide or CA repeats were amplified from 96 paired sets of tumour and normal tissue DNA using radioactive pcr. The amplification products were run on a 6% acrylamide urea gel for 2-3 hours. After separation the gels were dryed and exposed to X-ray films (Fuji) and Phosphor-Imager (Mol. Dynamics) systems. Allelic imbalance was defined as change in the relative intensity of the hybridization signal in tumour DNA when compared to the corresponding control DNA. Representative examples are shown in figure 1. The loci examined are on chromosome 14q: the SSTR1 and the B-myosin heavy chain (HMSYHCO1) locus[22]. And on chromosome 17q the SSTR2, the human growth hormone (GH)[23], and the myeloperoxidase glycoprotein (MPO) locus[24].

PCR conditions: denaturation at 94°C for 4 minutes, 25 cycles of denaturation at 94°C for 1.5 min, annealing at 55-60 °C for 1 min, extension at 72°C for 2 min and a final extension at 72°C for 10 minutes. Optimal buffer conditions were selected using the optimizer kit (Invitrogen). SSTR1 and GH: 300 mM Tris-HCl pH9.5; 75mM $(NH_4)SO_4$ and 7.5 mM $MgCl_2$. *SSTR2*, MPO and ß-MHC: 300 mM Tris-HCl pH9.5; 75mM $(NH_4)SO_4$ and 10 mM $MgCl_2$.

Results

Biotin-labelled phage clones containing the coding sequences of the human *SSTR*1, 2, 3 and 4 genes were mapped at chromosome bands 14q13, 17q24, 22q13.1 and 20p11.2, respectively on normal human lymphocyte metaphases (Fig 1a,1b).

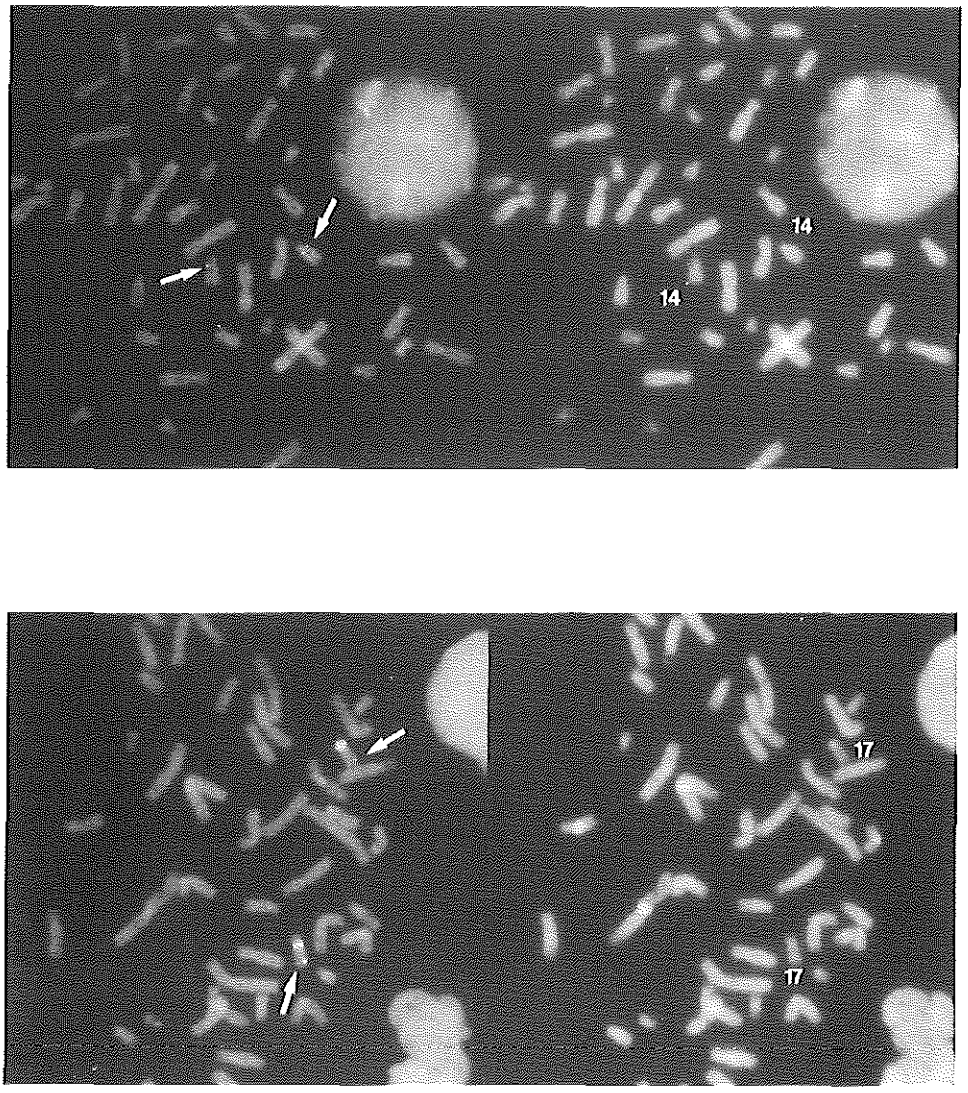

Figure 1: FISH signal obtained on chromosome 14q13 and on chromosome 17q24 using *SSTR*1 and *SSTR*2 probes, respectively. On the right DAPI staining of the same metaphases.

To investigate the number of chromosomal loci harbouring *SSTR1* or *SSTR2* sequences, metaphases of the two somatostatin receptor-positive breast cancer cell lines MCF-7 and ZR75-1 were hybridized with biotinylated *SSTR1* and *SSTR2* phage DNA. The results are shown in table I.

Table I: FISH-results from *SSTR1* and *SSTR2* on the SSTR positive breast carcinoma cell lines ZR75-1 and MCF-7.

Number of signals per metaphase with	MCF-7		ZR75-1	
A: *SSTR1*-probe				
1	0	0%	3	6%
2	9	14%	21	45%
3	40	65%	22	47%
4	13	21%	1	2%
B: *SSTR2*-probe				
1	0	0%	0	0%
2	6	50%	2	15%
3	6	50%	4	31%
4	0	0%	4	31%
5	0	0%	3	23%

The ploidy of the MCF-7 cell line ranges from hypertriploid to hypotetraploid (66-87 chromosomes). The ploidy of the ZR75-1 cells is near-triploid (modal number: 71 chromosomes). On the basis of these ploidy studies we would expect to find three to four hybridization signals in MCF-7 cells metaphases and three signals in ZR75-1 metaphases. In both cell lines the number of *SSTR1* hybridizing signals is as expected i.e. 3-4 in MCF-7 and 2-3 in ZR-75-1. Using the *SSTR2* phage probe, the cell line MCF-7 showed 2-3 hybridizing signals on chromosome 17q derivatives or marker chromosomes, which is a little less than expected. In metaphases of the cell line ZR75-1, 2-5 hybridizing loci were found on either chromosome 17q derivatives or on marker chromosomes. This is higher than expected on basis of the ploidy of the cell line.

Allelic imbalance of the somatostatin receptor 1 and 2 genes was assessed by amplification of the polymorphic CA repeats located in the vicinity of these genes. A high percentage of the tumours proved to be informative and allelic imbalance was detected in approximately half of these cases for both the *SSTR*1 and the *SSTR*2 loci (table II).

Table II: Allelic imbalance of chromosome 14q and 17q dinucleotide repeat polymorphisms.

CA-repeat	heterozygozity	allele size	allelic imbalance
*SSTR*1	84%	211-185 bp	50% (34/67)[a]
ß-MHC	82%	158-130 bp	33% (25/75)[a]
*SSTR*2	88%	148-132 bp	52% (40/77)[a]
GH	83%	253-201 bp	40% (13/32)[a]
MPO	55%	110-104 bp	46% (32/69)[a]

[a]: remaining cases not informative or not determined.

In some tumours allelic *SSTR* sequences were lost but in other samples gain of DNA sequences was observed leading to clear overrepresentation of one allele (Fig.2) Polymorphic CA repeats of the ß-myosin heavy chain (ß-MHC) gene on chromosome 14q and the growth hormone (GH) and myeloperoxidase (MPO) genes on chromosome 17q were investigated as well and served as indicator of the specificity of the imbalance of the somatostatin receptor genes. In general these genes showed the same pattern of allelic imbalance as was observed for the *SSTR* loci, albeit to a lower extent.

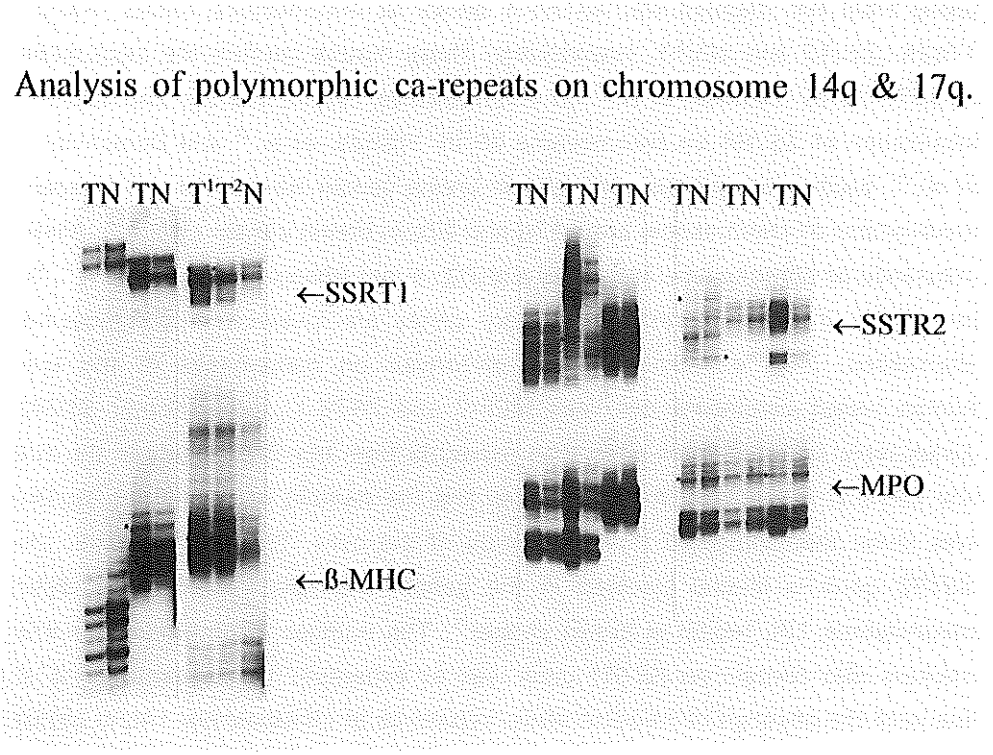

Figure 2: Autoradiogram showing the amplification patterns of the polymorphic CA repeats located in the vicinity of the *SSTR*1 and *SSTR*2 loci, and of the *ß-MHC* and *MPO* genes that were used as a control. Allelic imbalance is indicated by dots at the left hand side of the lane. TN: paired tumour and lymphocyte or skin DNA from the same patient.

Discussion

Using FISH we mapped the *SSTR*1-4 genes at chromosome bands 14q13, 17q24, 22q13 and 20p11, respectively. These results confirm the localization of *SSTR*1-3 by Yamada, *et al.*[21] on chromosomes 14, 17 and 22, and also the localization of *SSTR*4 on chromosome 20 by Yasuda, *et al.*[25] and Demchysin , *et al.*[10]. Studies on genetic changes in breast cancer have shown a frequent involvement of chromosome arms 17q and 22q in breast cancer cells[15,16]. Allelic imbalance of chromosome arms 14q and 20p is not observed frequently[15-17]. To investigate if the somatostatin receptor genes are involved in chromosomal aberrations, FISH experiments were performed on metaphases of the two human breast carcinoma cell lines MCF-7 and ZR75-1. Interestingly, *SSTR*2 genes were found at more sites than expected from the DNA ploidy in the cell line ZR75-1, whereas in the MCF-7 cell line the number of hybridizing signals was lower than expected from the ploidy of the cells. This suggests that the *SSTR*2 gene is indeed involved in the genetic changes involving the distal chromosome 17q region in breast cancer[14]. The number of chromosomes and markers that contain an *SSTR*1 gene was as expected. This observation is in line with the absence of frequent allelic imbalance of chromosome 14q in breast cancer[17].

To get a better understanding of the involvement of the *SSTR*1 and *SSTR*2 genes in human primary breast tumours, allelic imbalance of these genes was investigated in 96 breast carcinomas. In many of these breast tumours we observed allelic imbalance of the *SSTR*1 and *SSTR*2 genes (see table II). Imbalance of the *SSTR*2 locus on chromosome 17q again fits well with the frequently observed allelic imbalance of chromosome 17q[14-16]. However, the high frequency of allelic imbalance of chromosome 14q was not reported before[17]. In most primary breast cancer samples allelic imbalance was also observed at the other investigated loci on these chromosomes, suggesting that large chromosomal areas or whole chromosome arms are involved.

Whether the observed loss or gain of *SSTR*1 and *SSTR*2 sequences is functional and correlates with the pattern of SSTR expression in the tumour found by

autoradiography, is now under investigation. This and future studies of SSTR expression in breast cancers will help to determine the value of these parameters for the patient's prognosis and may probably serve to design more target specific therapies.

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CHAPTER IV: GENETIC ASPECTS OF BREAST CANCER

IV.1: RETINOBLASTOMA GENE ALTERATIONS IN HUMAN PRIMARY BREAST CANCER ARE RELATED TO SMALL TUMOUR SIZE AND ABSENCE OF LYMPH NODE METASTASIS.

Submitted.

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Abstract

Gene alterations of tumour suppressor genes and oncogenes are the fundamental changes that lead to tumour initiation and progression. In human breast cancer, alterations of the retinoblastoma (*RB*) tumour suppressor gene are frequently observed. In this study ninety-eight primary breast tumours were used to investigate the relation between structural alterations in the *RB* gene and clinical and pathological parameters such as patient age, nodal and menopausal status, stage, grade and steroid receptor status of the tumour. Using Southern blot analysis and two *RB*-cDNA probes spanning the *RB* coding region we were able to detect gross alterations or loss of an *RB* gene copy in 23 of the 96 primary breast tumours. From 93 of the patients clinicopathological data were available. Comparison of the observed alterations in the *RB* gene with the clinicopathological parameters revealed no significant correlation between *RB* alterations and patient age, menopausal status, differentiation grade or oestrogen receptor levels. However, a statistical significant correlation was observed between alterations in the *RB* gene and a small tumour size ($p < 0.01$), and between *RB* gene alterations and absence of lymphnode involvement ($p < 0.01$).

Introduction

Alterations in oncogenes and tumour suppressor genes are fundamental changes that may lead to a malignant phenotype. These genetic alterations are also present in human breast cancer. Since breast cancer is a very heterogeneous disease it is of particular interest to identify markers that discriminate between subgroups of breast cancer with a different biological and/or clinical behaviour. This may lead to better treatment strategies and furthermore may serve as a tool to delineate breast cancer heterogeneity[1]. Various genetic changes have been observed in human breast cancer. These include amplification of the *CMYC*, *INT2* and *HER2/NEU* oncogenes[2-5], as well as loss of heterozygosity (LOH), suggesting the involvement of a tumour suppressor gene, on

chromosomes 13q, 18q, 17p and 17q[6,7]. The retinoblastoma (*RB*) tumour suppressor gene is located on chromosome 13q14. Introduction of a wild type *RB* gene can reverse the neoplastic phenotype of some breast cancer cell lines[8]. Structural alterations in the *RB* gene have been detected in 19%-30% of primary breast tumours[7,9,10]. A similar incidence (15-29%) of loss of RB protein expression has been reported by Trudel, *et al.*[11], Varley, *et al.*[9] and Borg, *et al.*[10]. In the latter two studies no clear correlation between structural *RB* gene alteration and loss of RB protein expression was observed. Their results suggest that an alteration in the *RB* gene locus do not always lead to loss of RB protein, but can even be associated with an increased protein level. At present it is unclear whether the observed alterations in the *RB* gene are directly involved in breast tumour development or are merely secondary events. This latter possibility is strongly suggested by the failure to detect mutations in the remaining *RB* allele[12], the fact that LOH of chromosome 13q is frequently associated with allelic loss of other loci[13], aneuploidy of the tumour cells[10] and the exclusion of the *RB* gene as predisposing gene in familial breast cancer[14]. However, these observations could be biassed by the heterogeneity of breast cancer and it is well possible that loss of the *RB* gene plays a role in a specific subgroup of breast cancers. Obvious candidates are neuroendocrine breast tumours, since *RB* gene alterations have been frequently observed in other neuroendocrine tumours like small cell lung carcinoma[15]. However, we found no correlation between the expression of neuroendocrine differentiation markers and RB loss in a series of 49 tumours[16]. Another way to evaluate the involvement of *RB* gene alterations in subgroups of breast cancer is the investigation of possible associations between the genetic events and specific tumour or patient characteristics. Trudel, *et al.* reported a weak ($p = 0.045$) association between immunohistochemical presence of RB protein and grade three tumours[11]. Varley, *et al.* observed that the frequency of genetic changes in the *RB* gene increased with poorer differentiation and with increased spread of the disease[9]. These data suggest either an association with aggressive disease or involvement late in carcinogenesis. Complete comparison of the tumour and patient characteristics in the previous studies was not possible

because not all relevant data are presented in each separate study.
Here we report on associations between *RB* alterations in primary breast cancers and tumour and patient characteristics.

Materials and methods

Patient and tumour characteristics

Ninety-eight primary breast tumour specimens from patients (mean age: 59.6 years; range: 28-84 years) without signs of distant metastasis at the time of surgery were included in this study. These tumours were among those that were used in another study[4]. Data on tumour size, nodal status including the number of positive nodes, pathological grade of the tumours, steroid receptor status and age and menopausal status of 93 patients are shown in Table I.
The estrogen receptor (ER) and progesterone receptor (PR) were measured as recommended by the EORTC Breast Cancer Cooperative Group[17].

Southern hybridizations.

Three to eight μg DNA from each tumour sample and from one normal control DNA, was digested with restriction endonuclease *Hind*III, separated on a 0.8% agarose gel and transferred to Hybond N^+ nylon membranes (Amersham, Aylesbury, UK). The membranes were hybridized with ^{32}P-oligolabeled DNA probes. Hybridization and washing procedures were performed under standard conditions. After autoradiography on XAR films (Eastman Kodak, Rochester, NY) for 1 to 7 days, the membranes were stripped using 0.5% Na $DodSO_4$ (100°C, 15'). Two *Eco*R1 cDNA fragments of 0.9 (pGH2) and 3.8 kb (pG3.8M) representing the 5' and 3' *RB* cDNA respectively were used as probes[18]. A tumour was designated as harbouring an altered *RB* gene when the hybridization patterns with both 5' and the 3' *RB*-cDNA probes showed visually detectable

TABLE I: Relationship between *RB* gene alterations patient or tumour characteristics

Patient group	No. of patients	RB % altered	P value
Total	93	24	
Age (yr)			
<50	29	24	NS[a]
50-65	25	24	
>65	39	21	
Menopausal status			
Pre/peri	34	26	NS
Post	59	20	
Tumour stage			
pT1	19	53	<0.01
pT2	54	15	
pT3/T4	19	16	
pTx[b]	1	0	
Lymph node status			
pN0	24	46	<0.01
pN^+	69	14	
Differentiation grade			
Well	3	33	NS
Moderately	29	21	
Poorly	50	24	
Unknown	11	18	
Steroid receptor levels[c]			
ER-	25	12	NS
ER+	68	26	
PR-[d]	25	24	NS
PR+	62	23	

[a]NS, not significant; [b]Tx status unknown; [c]Cutoff points: 10 fmol/mg protein; [d]not performed on all cytosols .

alterations such as changes in the size of the hybridizing fragments and changes in the relative strength of the hybridization signals[16]. As control for the amount of DNA loaded on the gel the filters were hybridized with an IGF-1-Receptor probe (pIGF-9-R.8, ATCC 59295).

Statistics

The aim of the analysis was to study the associations between patient- and tumour characteristics and the occurrence of *RB* gene alterations. This was done by cross tabulation. Differences were tested with Pearson's chi-square test.

Results

Hybridization patterns

Ninety-eight primary breast tumours were evaluated for genetic alterations of the *RB* gene. To this end Southern blots containing *Hind*III digested tumour DNA and control DNA was hybridized with the 5' and 3' *RB*-cDNA probes pGH2 and pG3.8M, respectively. These cDNA probes encompass the entire coding region of the *RB* tumour suppressor gene and give, in *Hind*III digested chromosomal DNA, a number of hybridizing fragments (Figure 1A & 1B). The pattern of hybridization and the intensity of the hybridizing fragments of the different lanes containing tumour and control DNA were compared to detect rearrangements of the *RB* gene. To find loss of a complete *RB* gene copy, we compared the intensity of the hybridizing 5' and the 3' *RB* DNA fragments (Figure 1A and 1B, respectively) with the intensity of the hybridizing fragments obtained with the control probe (Figure 1C). Although this method will predominantly reveal large deletions and gross alterations of the *RB* gene and not the smaller type of mutation, we observed alterations in 23 of the 96 (24%) tumour DNAs (in two cases the difference in hybridization pattern was not conclusive). These two

samples and one other of which no clinical/pathological data were available were excluded from the statistical analysis.

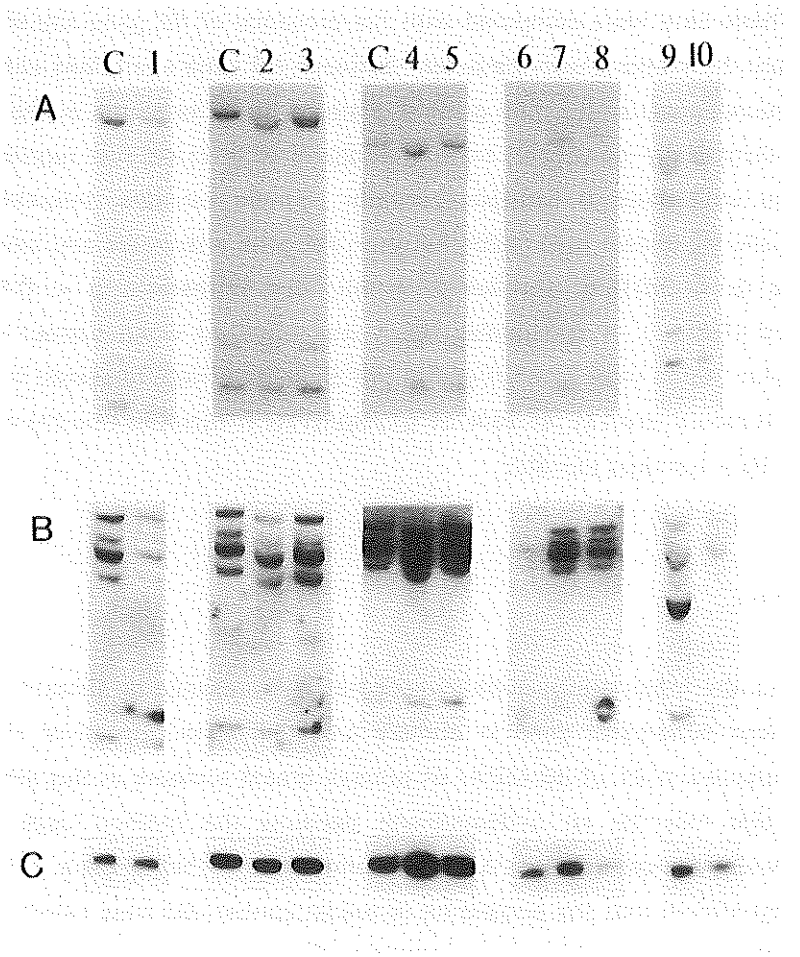

Fig. 1: *RB* alterations in primary breast tumour.
Southern blot analysis comparing the integrity and copy number of the *RB* gene in *Hind*III digested tumour DNA (lanes 1-10) and non-tumour control DNA (lanes C). The blots were hybridized with a 5' (panel A), 3' (panel B) *RB* or *IGF1-R* (panel C) cDNA probe. No *RB* alteration: DNAs lanes 3,5,8,10. Rearrangement of the *RB* gene: DNA lane 9. Loss of *RB* gene sequences: DNA lanes 1,4,6. In these lanes the intensity of the *RB* hybridization bands compared to the *IGF1-R* bands is to low. Less obvious is the loss of *RB* sequences in lanes 2 and 7. However, also in these lanes the signal intensity is reduced.

Relationship between *RB* alterations and patient and tumour characteristics.

Patient and clinical data were available of ninety-three of the remaining cases. When these data were compared with the presence of *RB* gene alterations, no significant correlations were detected between *RB* gene alterations and patient age or menopausal status, and grade and steroid receptor status of the tumour. A (not statistically significant) trend was observed towards over representation of these alterations in estrogen receptor positive tumours. Interestingly, a statistically significant association between *RB* gene alterations and small, pT1, tumours ($p < 0.01$) or absence of lymph node metastasis ($p < 0.01$) was observed (Table I). A comparable result was obtained when tumour size and lymphnode status were taken together: *RB* alterations were observed in 64% of pT1N0 tumours (n = 11), in 33% of pT2N0/pT1N+ tumours (n = 21) and 15% of pT > 2N+ tumours (n = 60).

Discussion

Using Southern blot analysis we investigated the presence of alterations of the *RB* tumour suppressor gene in primary breast cancers. Structural alterations were detected in 21 of 96 (22%) of primary breast tumours in this series. This percentage may be an underestimate because the method used, detects predominantly larger deletions and gross alterations in the *RB* gene. However, it has been shown that the second hit in breast cancer cell lines usually involves large deletions of the gene[19], or complete loss of a chromosome[20]. Both are detectable with the method used in this study. The results are comparable with the incidence of *RB* gene alterations reported on by others. With a similar approach, structural alterations in the *RB* gene were detected in 19-30% of the primary breast cancers[9,16]. In LOH studies 25% of the tumours showed loss of heterozygosity[1,10].

In most studies limited data on patient and tumour characteristics were available and a correlation of these data and *RB* gene alterations was not possible.

In our analysis of 93 cases, we found a correlation between *RB* gene alterations and a small tumour size ($p < 0.01$) and absence of lymph node metastasis in the patient ($p < 0.01$). The weak association with estrogen receptor expression was not statistically significant. These results suggest an early involvement of the *RB* gene in a subgroup of breast cancers with characteristics that favour a better prognosis. This is in contrast to the results from Varley, *et al.*[9] and Borg, *et al.*[10] that suggest an association with more advanced and aggressive disease. However, this difference could also be the result of differences in tumour populations and the techniques that were used. To get better informed about the significance of *RB* alterations for the prognosis of the patient we have subsequently collected follow-up data of the patients under investigation in the present study (see Appendix).

Acknowledgement

This study was supported by a grant (DDHK 92-04) of the Dutch cancer foundation (PB, WvP, JK), and by the Royal Dutch Academy of Science (AdK).

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IV.2: LINKAGE TO MARKERS FOR THE CHROMOSOME REGION 17q12-q21 IN 13 DUTCH BREAST CANCER KINDREDS.

American Journal of Human Genetics, 52: 730-735, 1993.

Am. J. Hum. Genet. 52:730–735, 1993

Linkage to Markers for the Chromosome Region 17q12-q21 in 13 Dutch Breast Cancer Kindreds

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Summary

We have performed linkage analysis with five markers for the chromosome region 17q12-q21 in 13 Dutch breast cancer kindreds in order to find support for the claim by Hall et al. that a gene in this region, termed "BRCA1," is associated with predisposition to early-onset familial breast cancer. This work is part of a collaborative study, the results of which are published elsewhere in this issue. Best evidence for linkage was observed with the marker CMM86 (D17S74) in pedigrees with an average age at onset of ≤47 years (LOD score = 1.77 at 1% recombination). In one breast-ovarian cancer family with a high probability of being linked to 17q, we observed one putative recombinant between D17S250 and D17S579, which suggests that BRCA1 is proximal to D17S579.

Introduction

Familial clustering of breast cancer has been observed in many epidemiological studies and may involve up to 15% of all investigated breast cancer cases (Lynch et al. 1984, 1989). However, a genuine hereditary component caused by a segregating predisposing gene has been proposed to be involved in no more than half of these (Lynch et al. 1989). The characteristics of hereditary breast cancer (HBC) include a relatively early mean age at onset, excess of bilaterality and multiple primary cancers, clear maternal or paternal transmission of the disease, and, in some families, preponderance of specific other types of malignancy. Most segregation analyses of such kindreds have indicated that an autosomal dominant susceptibility gene with incomplete penetrance may explain the various forms of familial clustering (Go et al. 1983; Goldstein et al. 1987; Bishop et al. 1988; Newman et al. 1988; Claus et al. 1991).

With the advent of multiallelic DNA markers, the power to detect linkage in breast cancer kindreds has increased significantly (Narod and Amos 1990). One such a marker, defining the locus D17S74 at 17q21, yielded conclusive evidence for linkage with familial early-onset breast cancer (Hall et al. 1990). In those pedigrees that showed a positive LOD score, breast cancer was the preponderant malignancy, and, with one exception, the average age at onset was ≤47 years. It is interesting that this linkage was also found in a number of breast cancer pedigrees showing a concurrent increased incidence of ovarian carcinoma (Narod et al. 1991).

A collaborative group aimed at identifying linkage in HBC was formed in 1989 (Lenoir et al. 1990). This consortium has recently been investigating the critical 17q-region in a total of 214 families (Easton et al. 1993). We report here the Dutch contribution to this data set, which comprises 13 breast cancer families, studied at five markers for the region 17q12-q21.

Subjects, Material, and Methods

Family Resources

Families used in this study were contributed by three centers: three families were reported by The Netherlands Cancer Institute, Amsterdam; one family was reported by Erasmus University, Rotterdam; and nine families were reported by the Foundation for the De-

Received June 15, 1992; revision received October 30, 1992.

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0002-9297/93/5204-0010$02.00

Table 1

Cancer Incidence in 13 Dutch HBC Families

Family	No. of Individuals	No. of Breast Cancer Patients	No. Verified by Pathology Records	No. of Patients under Age 47 Years[a]	Mean Age at Onset[b] (years)	Other Malignancies Verified by Pathology Records
1	65[c]	9	9	3	51	
2	15	6	4	⩾3	?	
3	22	6	5	5	41	1 Liver ca; age unknown
4	12	5	5	2	54	
5	51	5	4	3	43	
8	13	3	2	0	62	2 Seminomas; ages 30 and 31 years
10 ...	50	7	3	⩾1	?	1 Grawitz tumor; age unknown 1 Colon ca; age unknown
11 ...	36	3	3	2	47	1 Non-Hodgkin lymphoma; age 20 years
12 ...	21	4	4	3	49	
13 ...	28	4	3	2	55	
14 ...	19	3	2	2	46	1 Lymphatic leukemia; age unknown
16 ...	30	5	4	3	47	2 Ovarian ca; ages 48 and 60 years
19 ...	28	2	2	1	47	2 Ovarian ca; ages 45 and 52 years

[a] From pathology records or family interview.
[b] Breast and ovarian carcinoma patients; a question mark indicates that a few ages at onset of either breast or ovarian cancer were unknown.
[c] A smaller pedigree consisting of 10 individuals plus 4 breast cancer patients was analyzed with 17q markers.

tection of Hereditary Tumors, Leiden. Kindreds were selected on the basis of the presence of breast cancer or ovarian carcinoma in at least three first-degree relatives from at least two generations. These criteria were met in 10 pedigrees; 3 additional pedigrees were included because they had at least four breast or ovarian carcinoma patients in a single sibship. Of the 66 breast or ovarian cancer patients present in these pedigrees, pathology records were available for 53. In all others, status was ascertained by family interview.

Markers

The markers used in this study are the microsatellites Mfd15 (D17S250; Weber et al. 1990), Mfd188 (D17S579; Hall et al. 1992), 42D6 (D17S588; A. R. Oliphant, personal communication), and GH (Polymeropoulos et al. 1990). An RFLP was detected with probe CMM86 (D17S74), which was obtained from the American Type Culture Collection. Together these markers cover the region 17q12-q21. The assumed order of markers was D17S250–(8.1/3.3)–D17S579–(9.9/5.0)–D17S588–(17.7/7.1)–D17S74–(6.8/5.0)–GH, where the numbers in brackets refer to the genetic distance, in centimorgans, as observed in females/males (Easton et al. 1993).

DNA Isolation and Marker Analysis

Human genomic DNA was prepared from 40 ml heparinized blood samples, according to a method described elsewhere (Miller et al. 1988). For CMM86 RFLP detection, 5 μg of DNA was digested with *Hinf*I according to the supplier's protocols. Agarose gel electrophoresis, Southern blotting, probe labeling, and hybridization conditions have been described elsewhere (Devilee et al. 1989). Microsatellite polymorphisms were revealed by amplifying the region from 100 ng of genomic DNA, by using PCR in the presence of α-^{32}P-dCTP (Amersham). PCR conditions were essentially those described by Weber and May (1989). PCR products were separated on a standard sequencing apparatus (model S2; BRL), after which the gels were dryed and exposed to Kodak XAR films at −70°C.

Statistical Analysis

All marker data thus obtained were submitted on disk, in conjunction with the pedigree data, to the central data-handling facility, run by Drs. T. Bishop and D. Easton. All statistical methods are described in an accompanying paper (Easton et al. 1993) in this issue of the *Journal*. Parameters for gene penetrance and frequency were essentially those described by Claus et al. (1991).

Results

Description of Families

Table 1 lists the number of breast cancer patients in each family, the number of patients with an age at inci-

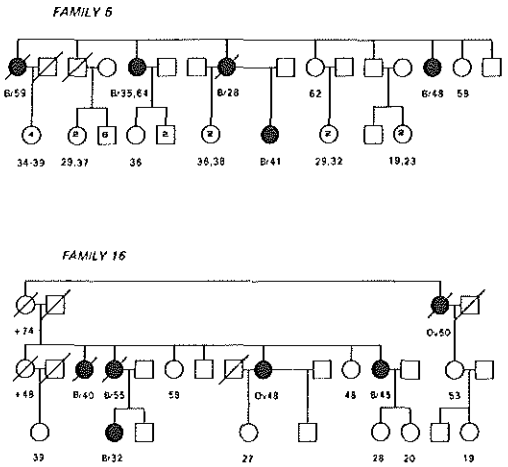

Figure 1 Representative examples of the pedigrees under study. Squares denote males; and circles denote females. Numbers within symbols are the number of individuals in the sibship. Blackened circles represent breast or ovarian carcinoma patients. Age at onset is given: e.g., Br59 = verified breast carcinoma at age 59 years; and Ov60 = verified ovarian carcinoma at age 60 years. Current ages are given only for healthy women who are part of the genealogy. A diagonal line through a symbol indicates that individual is deceased. For women who have not died from breast or ovarian cancer, the age at death is given as, e.g., "+48." The probability of linkage to 17q was .93 and .73 for families 5 and 16, respectively (Easton et al. 1993).

dence ≤47 years, and the mean age at onset. Other verified cancers appearing in the family are also listed. The 13 families comprise 339 individuals (215 females), from 247 of whom we were able to obtain blood samples. Each pedigree contains at least three breast and/or ovarian carcinoma patients. Two kindreds (16 and 19) were classified as breast-ovarian; all others were considered site-specific breast cancer families (for classification, see Lynch et al. 1989; Easton et al. 1993). A total of 66 breast or ovarian carcinomas were reported in these 13 families. The mean age at onset was ≤47 years in six families, >47 years in five families, and, because of unretrievable pathology records, undetermined in two families. The youngest generation was often significantly less informative, since virtually all these women were <35 years of age at the time of visitation and were generally free of disease. Most patients from the oldest generation generally were deceased and hence unavailable for genotyping. Two representative examples of the pedigrees under study are shown in figure 1.

Linkage Analysis

Cumulative pairwise LOD scores obtained with the markers D17S250, D17S579, D17S588, D17S74, and GH are listed in table 2. Over all families, none of the LOD scores reached the commonly accepted critical value of 3.0. A value of 1.53 was obtained with D17S74 at $\theta = .10$. The highest individual LOD score was 0.97 at $\theta = .001$, obtained with D17S579 in family 5 (not shown).

We then divided the pedigrees into two groups, one with an average age at onset ≤47 years and one with an average age at onset >47 years. It is interesting that all markers then gave higher LOD scores at smaller θ values in the earlier-onset families, relative to the total cumulative LOD score—although, again, none surpassed the critical value of 3.0. In addition, the later-onset families showed lower LOD scores relative to all families. The best evidence for linkage was obtained with D17S74—i.e., a LOD score of 1.77 at $\theta = .01$—followed by D17S250—i.e., 1.38 at $\theta = .001$ (table 2).

While this work was in progress, it became apparent that the most probable location for BRCA1 was proximal to D17S74, between GIP and D17S250 (Hall et al. 1992). Analysis of the families present in the consortium by using a number of markers for this region identified several probable recombinants, confirming D17S250 as a proximal marker and revealing D17S588 as the closest distal border (Easton et al. 1993). Therefore we haplotyped all pedigrees for the marker order (cen)–D17S250–D17S579–D17S588–D17S74–(tel). In family 19, a breast-ovarian family in which the estimated posterior probability of being linked to 17q was .75 (Easton et al. 1993) and in which the maximum LOD score was 0.82 at D17S250 ($\theta = .001$), the resulting haplotype revealed an interesting recombination event (fig. 2). The grandpaternal haplotypes had to be inferred, as was the haplotype of the woman deceased from ovarian carcinoma (Ov45). In addition to three crossovers placing BRCA1 proximal to D17S74, one putative recombination occurs between D17S250 and D17S579 in a woman with an ovarian carcinoma diagnosed at age 52 years. The mean age at onset in this family is 47 years. Although the occurrence of this many recombination events in a small chromosomal region in this small sibship is a fairly surprising event, there is some evidence to suggest that all crossovers occurred during female meioses (see legend to fig. 2). Female recombination is twice as frequent as male recombination, in this region, and the female distance between D17S588 and D17S74 is nearly 20 cM (Easton

Table 2

Pairwise Cumulative LOD Scores for BRCA1 and Chromosome 17 Markers

Locus and Family Group[a]	LOD Score at θ = .001	.01	.05	.10	.20	.30
D17S250:						
All	1.04	1.06	1.11	1.10	.94	.65
Age at onset ≤47 years	1.38	1.36	1.28	1.17	.91	.62
Age at onset >47 years	−.58	−.54	−.39	−.27	−.13	−.06
D17S579:						
All	−.56	−.41	.01	.30	.50	.43
Age at onset ≤47 years	−.14	−.03	.25	.39	.42	.32
Age at onset >47 years	−.74	−.71	−.57	−.41	−.17	−.05
D17S588:						
All	.60	.64	.79	.92	.94	.74
Age at onset ≤47 years	1.06	1.04	.97	.87	.65	.41
Age at onset >47 years	−.49	−.45	−.29	−.12	.09	.16
D17S74:						
All	1.26	1.31	1.47	1.53	1.37	.99
Age at onset ≤47 years	1.76	1.77	1.77	1.67	1.31	.87
Age at onset >47 years	−.73	−.70	−.52	−.34	−.12	−.02
GH:						
All	.06	.12	.34	.50	.58	.46
Age at onset ≤47 years	.56	.57	.58	.56	.45	.28
Age at onset >47 years	−.80	−.75	−.59	−.44	−.24	−.11

NOTE.—Female θ = 2 × male θ.

[a] Families with mean age at onset ≤47 years were HBC3, HBC5, HBC11, HBC14, HBC16, and HBC19; and families with mean age at onset >47 years were HBC1, HBC4, HBC8, HBC12, and HBC13.

et al. 1993). If it is assumed that the disease travels with the haplotype 1-4-8-3, this suggests that BRCA1 maps proximal to D17S579.

Discussion

The genotype data obtained with 17q markers on 13 Dutch breast cancer pedigrees presented here are part of a coordinated effort aimed at identifying predisposing genes in these cancer-prone kindreds (Lenoir et al. 1990; Easton et al. 1993). After the initial report of linkage between early-onset breast cancer and the marker D17S74 (Hall et al. 1990), and after the extension of this finding to breast-ovarian families (Narod et al. 1991), the consortium sought to confirm this linkage in the total collection of pedigrees. All the pedigrees contributed by us contained at least three first-degree relatives with breast or ovarian cancer. With two exceptions, all pedigrees had at least two patients with an age at onset ≤47 years.

Although the informativity of some of these pedigrees was low, we observed a strong suggestion of linkage with the marker D17S74 in a subgroup of earlier-onset families. This finding compares well with results presented by Hall et al. (1990). Further, we observed one recombination event between D17S250 and D17S579, suggesting that BRCA1 is proximal to D17S579. This family has a high probability of being linked to 17q, first because it is a breast-ovarian family for which heterogeneity results have suggested that most are 17q linked (Easton et al. 1993) and, second, on the basis of multipoint analysis (estimated posterior probability of .75). Thus the gene region would be significantly reduced if this recombinant is taken as being informative for its localization.

Our linkage results indicate that, despite the presence of genetic heterogeneity, an unknown number of phenocopies, and a gene with incomplete penetrance, a traditional gene search program in hereditary breast cancer by using linkage analysis can be successful, if the markers are of sufficient informativity and are close enough to the gene. In this regard, it is noteworthy that our collection of pedigrees reflects very well what is most often encountered in clinical settings with respect to familial cancers—i.e., deceased upper generations and too-young-to-be-informative lower generations.

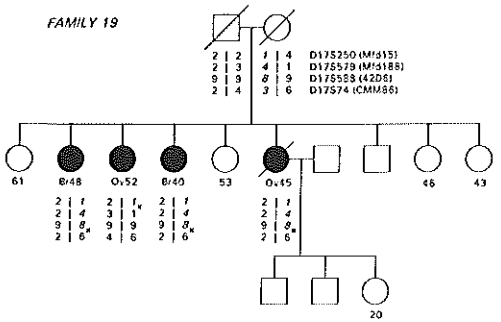

Figure 2 Haplotype of family 19 for the linked markers D17S250–D17S579–D17S588–D17S74. Symbols are as in fig. 1. Genotype data were obtained from all living members from this pedigree. Haplotypes from deceased individuals were inferred. All haplotypes other than the ones shown required that double recombinants be invoked. The presumed disease haplotype 1-4-8-3 is represented in italic characters. × = presumed meiotic crossover. Genotypes at D17S250 and D17S588 of both a sister of the grandmother and of a brother of the grandfather are consistent with the interpretation that the 1-4-8-3 haplotype derives from the grandmother (not shown).

If BRCA1 is a classical tumor suppressor gene for which Knudson's (1989) two-hit model is valid, then its involvement at the somatic level may be expected to be signified through loss-of-heterozygosity (LOH) mechanisms in hereditary as well as sporadic breast tumors. LOH on 17q in sporadic tumors has been found in several studies, in 30%–50% of informative cases (Cropp et al. 1990; Devilee et al. 1990, 1991; Sato et al. 1990). It is interesting that we observed that LOH at D17S74 was inversely correlated with the age at onset in the patient (Devilee et al. 1991). However, most investigators have used markers for the distal half of 17q to study LOH, usually D17S74 and D17S4. Our LOH mapping data indicate that, in some tumors, this region may undergo LOH without affecting the proximal part of 17q (Cornelis et al., in press). It has been suggested that NM23 (Leone et al. 1991) or the prohibitin gene (Sato et al. 1992) may serve as a target for these events. Since LOH often affects large parts of 17q, it may be difficult to identify which of the candidate genes—i.e., BRCA1, NM23, prohibitin, or even p53 on 17p13—has driven chromosome 17 LOH in each tumor. To this end, a high-density LOH map of 17q, constructed from hereditary as well as sporadic breast tumors, is needed. Such a study may include ovarian tumors as well, since 17q shows LOH in 65% of informative cases when the marker D17S74 is used (Foulkes et al. 1991).

Breast carcinoma is the most frequently occurring malignancy in women living in Western industrialized countries (Hirayama 1989), among whom it accounts for about 20% of all cancer deaths. Nevertheless, its cause remains elusive. Epidemiological studies have revealed several risk factors, including environmental influences (Willett 1989), age, history of breast cancer or proliferative disease (Dupont and Page 1985), and a positive family history (Lynch et al. 1989). Clearly, the precise localization and isolation of a predisposing gene will enable us to dismiss high-risk-family women from their 50% chance of being a gene carrier, as well as identify, with >95% probability, those at risk. However, it should be kept in mind that counseling on the basis of BRCA1 analysis will apply to only 5%–8% of all breast cancer patients (Lynch et al. 1989) and their relatives. In addition, the outlook, on either accurate early detection of breast cancer in women <50 years or breast cancer prevention, is unclear at present. In light of the mere 40%–50% 8-year survival rate under current therapeutic regimes (Yancik et al. 1989), we can only hope that the eventual understanding of the biology of BRCA1 function will provide us with clues for prevention or effective treatment for those at risk (Ponder 1991).

Acknowledgments

The authors thank Mrs. C. M. van der Drift for family administration. This work was supported by Royal Dutch Cancer Society project IKW 91-05.

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CHAPTER V: DNA REPAIR AND BREAST CANCER

V.1 THE INDUCTION OF ENHANCED REACTIVATION OF HERPES SIMPLEX VIRUS TYPE I IN SKIN FIBROBLASTS FROM A DUTCH BREAST CANCER FAMILY.

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Abstract

High levels of the SOS-like response enhanced reactivation of UV-irradiated herpes simplex virus type 1 (HSV1) in UV-irradiated cells have recently been shown in various hereditary cancer prone syndromes, such as Von Hippel Lindau disease and polyposis coli. This observation suggests that common mechanisms might be involved in carcinogenesis. Recently, similar results have also been demonstrated in the Li-Fraumeni syndrome. This cancer prone syndrome in which one allele of *P53* is mutated, shows a preponderance of breast cancer and various other types of malignancy. The identification of this and several other breast cancer syndromes raised the question whether a common pathway was involved in malignant transformation of breast cells. In this chapter we report upon an investigation of the induction of the reactivation of HSV-1 in UV-treated skin fibroblasts from members of a breast cancer prone family showing linkage to the *BRCA1* gene on chromosome 17q. In contrast to the high levels observed in other hereditary cancer prone syndromes, including the Li-Fraumeni syndrome, moderate levels of enhanced reactivation of HSV-1 were observed in this family, suggesting that different mechanisms might be involved in carcinogenesis in this family. Interestingly, the fractional survival of HSV1 in irradiated fibroblasts derived from this family is lower than in control cells, suggesting a DNA repair defect.

Introduction

Familial clustering of breast cancer is observed in approximately 15% of all breast cancers[1]. In about a third of these families there is evidence for a genetic predisposition to breast cancer. Based on the associated types of cancer that cluster in the syndrome that predisposes to breast cancer, four hereditary breast cancer syndromes have been defined[1]. In two of these syndromes the predisposition to breast cancer is linked to a gene locus: It has been proved that the Li-Fraumeni syndrome, in which breast cancer is associated with childhood sarcoma, brain tumours and adrenocortical carcinomas[2], is linked to a germ-line mutation in the *p53* gene[3]. In about 80% of the breast-ovary cancer families and 40% of the site-specific breast cancer families, linkage to the still hypothetical *BRCA1* gene on chromosome 17q21, has been found[4]. In the remaining families, which do not show linkage to either the *P53* or the *BRCA1* loci on chromosome 17, other genes predisposing to breast cancer must be involved.

At the cellular level cancer is the result of a dysregulation of cell growth. This dysregulation might be the result of mutations in growth regulatory genes. These mutations could lead to altered gene function, altered cell growth and ultimately to cancer. Mutations can be induced in the DNA by for instance chemicals or UV irradiation and exposure to either of these factors is not entirely preventable. DNA repair mechanisms normally remove introduced DNA damage before it can become hazardous. It has been shown that defects in DNA repair mechanisms, such as defects in excision repair mechanisms in xeroderma pigmentosum, lead to increased cancer incidence. Recently, evidence has been put forward that a defect in the DNA mismatch repair mechanism might be involved in the etiology of hereditary nonpolyposis coli (HNPPC)[5-7]. In earlier studies it was noticed that these tumours are characterized by variations in the length of dinucleotide repeats. It has been shown that HNPPC is linked to at least two loci, one on chromosome two and the other on chromosome three. Recently, the involved genes *hMSH2* and *hMLH1* have been identified as human homolog of the *MutS* and *MutL* genes that are involved in DNA mismatch repair

in bacteria and yeast. Interestingly, variations the length of trinucleotide repeats have been observed in some breast cancers[8]. This observation suggests that an analogous defective system is involved in breast cancer.

Another observation suggesting the involvement of an inducible DNA repair mechanism in familial cancer syndromes has previously been reported[9-11]. In these reports induction of the SOS-like response has been described in UV-treated skin fibroblasts from various familial cancer syndromes. The level of this induction can be calculated by an assay in which the survival (also called reactivation) of Herpes Simplex Virus type 1 is determined in UV exposed skin fibroblasts from members of cancer-prone families and compared to the survival in untreated cells (see material and methods). In cells from various hereditary cancer syndromes enhanced reactivation of Herpes Simplex Virus type 1 (ERHSV1) has been measured. Subsequently this SOS-like response has been related to disease expression in these families. Recently, high levels of the ERHSV1 response have also been found in cells from families with the Li-Fraumeni syndrome (Abrahams, personal communication). Here we report on an investigation of ERHSV1 in a 17q21-linked breast cancer family. Interestingly, recent observations in fibroblasts from various cancer-prone syndromes showed a abnormally decreased survival of HSV1 after UV irradiation of these cells, which is suggestive of a DNA repair defect. Here we report on our first experiments of these survival studies in the afore mentioned breast cancer family.

Material and methods

Description of the family: Of ten siblings, 3 male and 7 female, 5 females developed breast cancer of whom one (proband no. 7 in figure 1) bilateral breast cancer. The mean age at onset was 54 (range: 45-66). Their father (proband no.1) died from colon carcinoma. His sister died from breast cancer and one of his two brothers from lung carcinoma. The induction of the ER-response has been determined in all members of the nuclear pedigree shown in

figure 1 of whom informed consent could be obtained. The study was approved by the medical ethics comity of the Academic Hospital Rotterdam. No material was available from the deceased probands 1, 7 and 10.

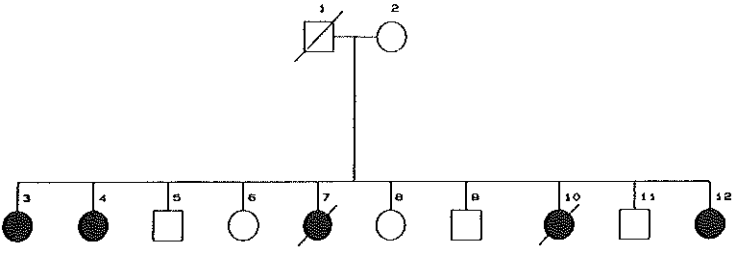

Fig 1: Nuclear pedigree of a breast cancer family. An open square or circle symbolize an unaffected male or female, respectively. A filled symbol indicates an affected proband. A line through the symbol indicates the proband deceased.

Linkage analysis: This family was included as family number four in the study described in chapter V.7[12]. The lod scores for this family are listed below.

Two point Lod-scores:

Marker/*	0.001	0.010	0.050	0.100	0.200	0.300
D17S250	0.00	0.00	0.00	0.00	0.00	0.00
D17S597	0.23	0.22	0.19	0.15	0.09	0.04
D17S588	0.37	0.36	0.33	0.28	0.18	0.09
D17S74	-0.27	-0.25	-0.20	-0.15	-0.07	-0.03

*:recombination fraction

Cell strains: Diploid fore-arm skin fibroblasts derived from three affected and six not affected members of the breast cancer family were grown in Ham's F-10 medium (Gibco Laboratories, Inc.) supplemented with 10% fetal calf serum.
VH10 normal diploid skin fibroblasts were used as indicator cells in the infectious centre assay[9].

ERHSV1 was measured in an infectious centre assay (see below).
To this end wild type HSV-1, Glasgow strain 17 syn^+, was grown as described[9]. The experiments were carried out with the same virus stock used in previous experiments[11]. The virus was exposed to a UV dose of 150 J/m^2. Not irradiated virus was used as a control.
Description of the infectious centre assay: Cell cultures were irradiated with 10, 15, 20 or 25 J/m^2, respectively. The virus was irradiated with 150 J/m^2. Twenty-four hours after the irradiation of the cells confluent cultures of family member skin fibroblasts in 6-cm petri dishes, were rinsed with phosphate buffered saline, inoculated with 0,2 ml of either irradiated or unirradiated virus suspension and placed at 37°C for 1,5 h, with occasional agitation of the cultures. In order to start the replication of the virus, the cultures were incubated for another 1,5h under liquid holding conditions (medium with 2% serum) at 37°C. The infected cells were trypsinized, mixed with 10ml of a suspension of uninfected VH10 indicator cells ($\pm 10^6$ cells per ml), and seeded onto 6-cm petri dishes. The cells were allowed to attach for about 2-3 hours at 37°C. After removal of the medium, 5 ml of agar overlay was added. The cultures were incubated at 37°C during three days, and stained with 0,00125% neutral red when the cytopathogenic effect was visible as plaques.

All these experiments were carried out in fourfold. The number of plaques was counted by two individuals on two occasions. The mean of each set of four experiments was then calculated. ERHSV1 is the ratio of the survival of UV-irradiated virus in UV-irradiated cells over that in unirradiated cells. Under these conditions a series of normal human diploid skin fibroblasts showed ER levels between 1,7 and 2,7[10]. In the present study this range of ER levels has been used as controls.
Fractional survival of unirradiated HSV1 in fibroblasts that were irradiated with several UV dosages were investigated in a similar infectious centre assay.

Results

ERHSV-1 in diploid forearm fibroblasts has been determined using an infectious centre assay in a family with hereditary breast cancer. The investigated family showed probable linkage to the *BRCA1* locus on chromosome 17q.
The absolute number of plaques depended on the UV dose exposed to the cells as is also observed in normal control cells. Usually the highest number of plaques can be observed in the dishes exposed to 10 and 15 J/m^2 and a decrease at the higher levels. The latter suggest a toxic effect of these higher UV doses. Interestingly, it was noticed that certain cell lines exhibit a dramatic drop of the plaque forming ability after UV-exposure even at lower UV-dosages. This observation suggested that the repair capacity might be impaired in these cells. To investigate this, the survival of UV-irradiated HSV-1 in some family members has been determined. As is shown in figure 2 the survival of HSV1 in fibroblasts from family members, both affected and not-affected, is indeed lower than in the control cells. Remarkable is that the survival is also decreased in the cells from a sporadic breast cancer patient.

TABLE I: Fractional survival of UV-C irradiated HSV1 in human fibroblasts

Results in a breast cancer family

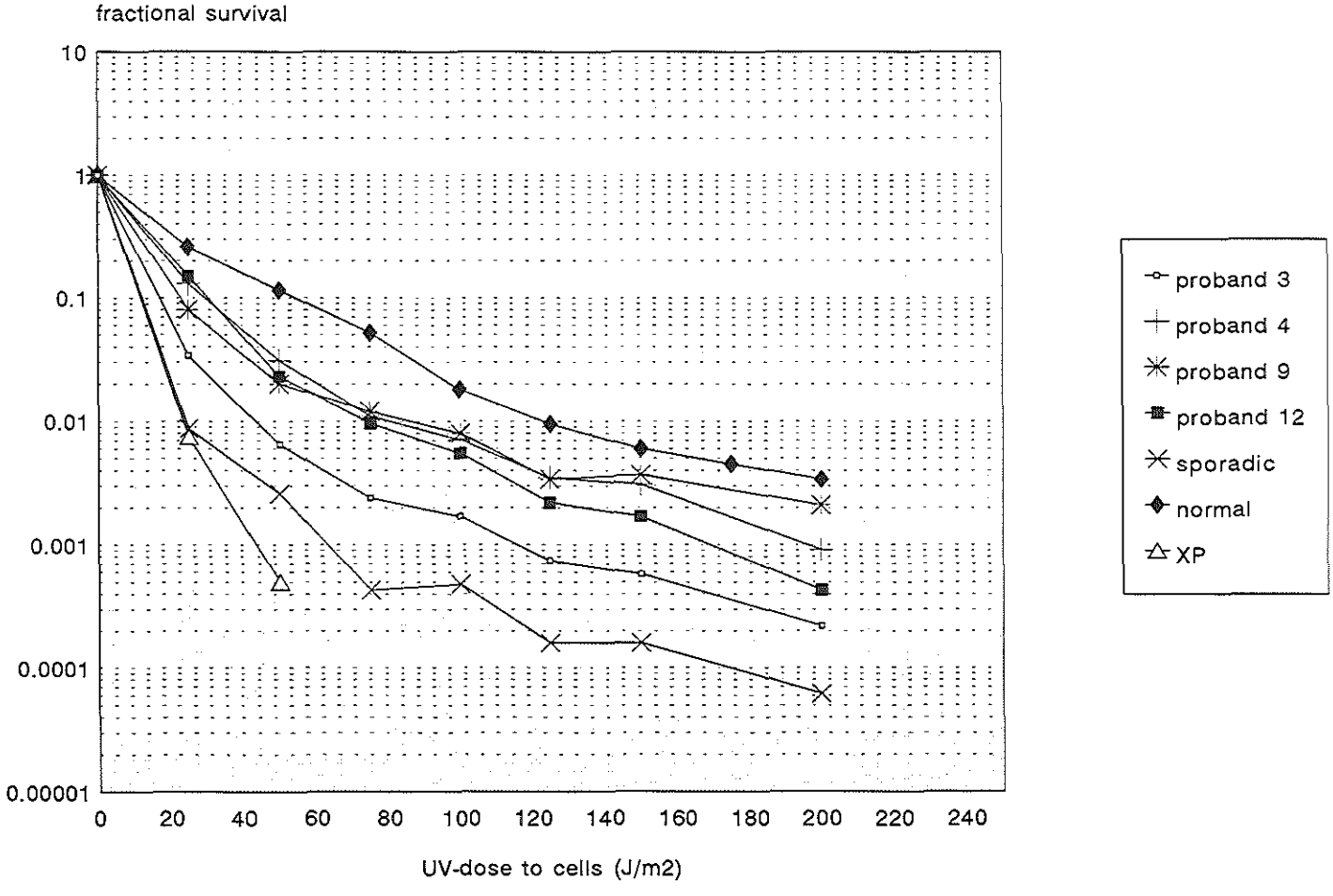

TABLE I: Enhanced reactivation of HSV-1.
The numbers above the columns correspond with the numbers of the probands in figure 1. ERHSV1 = enhanced reactivation (see material and methods). ERHSV1 was measured using an infectious centre assay as described under material and methods.

UV dose to	ERHSV1								
cells (J/m^2)	2	3	4	5	6	8	9	11	12
0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
10	2.3	1.1	-	1.5	1.2	2.1	2.1	1.4	2.4
15	2.9	2.4	3.4	3.5	2.4	7.2	3.6	2.8	2.7
20	4.3	9.2	3.5	2.2	4.4	8.0	4.8	3.6	4.0
25	3.0	4.0	5.8	1.7	5.4	8.0	3.4	2.9	3.4

ERHSV1 was observed in all individuals tested (Table I). It can be seen that the ERHSV-1 in some cell lines is high, whereas in others it is comparable to that in normal cells. In contrast to previous observations in other cancer-prone syndromes none showed the high levels (> 10) of ERHSV1 that have been observed in for instance Von Hippel Lindau and polyposis coli.
Moderately elevated levels were observed in some affected and also in some non-affected family members. Based on the higher levels of individuals 3 and 4 it is suggestive that individuals 6 and 8 might possibly be carriers.

Discussion

Induction of ERHSV1 has been investigated in a family with a predisposition to breast cancer. Previous investigations had shown probable linkage of the breast cancer predisposition in this family to the *BRCA1* gene on chromosome 17. Moderate levels of ERHSV1 were detected in this family, both in patients and healthy family members. A clear correlation with expression of the disease was therefore not established. However, the higher ERHSV1 levels of individuals 3 and 4 suggest that the unaffected individuals 6 and 8 might be carriers. Similar

results were obtained with cells from unaffected individuals from a Lynch type II family (Abrahams, personal communication). Clinical follow-up will show if the ER-response might be used as a prognostic marker. Interestingly, our preliminary results on the absolute survival of virus in the cells suggest that this survival was lower than in normal cells (Fig 2) . A similar observation was made in several other cancer-prone syndromes (data not shown), and also in a case of sporadic breast cancer. This could be an indication of a lower or slower overall level of DNA repair. Recent investigations have coupled slower DNA repair to a higher frequency of induction of mutations in the regions of slower DNA repair: mutation hot-spots in the *p53* gene show no DNA repair activity[13,14].

The accumulation of genetic alterations is the fundamental genetic process that can eventually lead to malignancy. Like in the syndromes coupled to defects in the nucleotide excision repair (NER) and mismatch repair systems.

In the investigated cancer families also a lack of repair was observed. The enhanced induction of SOS-repair might be a physiological response to a lack of negative feedback by other (defective) repair systems. The observation that various familial cancer syndromes, caused by different germ-line mutations show this response, suggests that the predisposing genes have multiple functions. They apparently share a function in DNA repair, other functions lead to the associated cancer syndrome. Simultaneously this implicates that only accumulation of DNA mutations by lack of DNA repair is not sufficient for the development of a familial cancer syndrome. This is yet another example of the accumulating evidence of the involvement of different DNA repair mechanisms in the etiology of cancer. Other examples are the defective nucleotide excision repair (NER) in xeroderma pigmentosum, the linkage of hereditary nonpolyposis coli to genes involved in a DNA mismatch repair system, and the increased incidence of breast cancer by an unknown mechanism in heterozygotes for the ataxia telangiectasia gene. Many of these diseases have a clinically heterogeneous pattern of expression. In the case of the NER syndromes it has been proved that in several cases, the defective gene has a dual function. It is involved in DNA repair as well as in the basal transcription mechanism, an essential process in all cells[15]. This explains both the rarity of these syndromes

and the clinical heterogeneity concerning photosensitivity, neuronal degeneration, growth abnormalities etc.

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CHAPTER VI: GENERAL DISCUSSION AND CONCLUDING REMARKS

The experimental work described in this thesis aimed at delineating the heterogeneity of breast cancer in an attempt to increase our understanding of the significance of genetic alterations in breast cancer. To this end we investigated three subgroups of cancer: Primary breast cancers that express the somatostatin receptor, primary breast cancers with alterations in the *RB* gene, and families that show a predisposition to breast cancer. Although this approach turned out to be fruitful, many questions remained and many new interesting topics that warrant further research appeared during our experiments. In this sections some of these topics are addressed.

Expression of somatostatin receptors in primary breast tumours was investigated using *in-vitro* autoradiography (III.1) and *in-vivo* scanning (III.2). Both methods have their own characteristics. *In-vitro* autoradiography has the advantage of directly correlating somatostatin receptor expression to tissue histology. This is especially important in tissues, like breast cancer that contain areas of somatostatin receptor positive and somatostatin receptor negative cells. *In-vivo* scanning has the advantage that somatostatin receptors can be identified in patients without a prior need for operation. Furthermore the sensitivity of the method is higher than that of *in-vitro* autoradiography because it registrates the somatostatin receptors three dimensionally in the entire tumour. Clinical applicability of somatostatin and its receptors was enhanced by this technique as illustrated by the possibility to detect metastases of somatostatin receptor positive tumours with much higher sensitivity than with other radiological techniques. At present the identification of a distant metastasis usually implicates that the disease is not curable and that the patient receives palliative treatment. Early identification, and the perhaps the future possibility of local radiotherapy using somatostatin analogs that are coupled to beta-wave isotopes might prove a valuable new attempt to cure these patients from their disease. Theoretically, early detection might also beneficial in a palliative setting since it is well known that chemotherapy is more effective in

patients with a low tumour burden, although "low" has not been defined.

A positive influence of somatostatin receptor expression on the prognosis of the patient was indirectly suggested by the correlation of SSTR expression with small tumour size. This positive influence is now questioned by the data presented in chapter III.2. These data show that 11 of 39 (28%) patients with a SSTR-positive tumour without metastases at the time of diagnosis, have developed distant metastases within two and a half years of follow up. This is highly suggestive of aggressive disease. However, this suggestion has first to be validated by data on patient survival, before conclusions on the prognostic significance of SSTR expression in breast cancer can be reached. A major problem in the interpretation of these studies is the lack of a marker in the SSTR negative tumours.

An ongoing debate is that about the origin of somatostatin receptor-positive breast tumours. Is somatostatin receptor expression a constitutional characteristic, already present before the cells become malignant, or alternatively is SSTR expression an acquired characteristic expressed during malignant transformation. Part of the experimental work described in this thesis is based on the assumption that breast tumours that express somatostatin receptors, are a specific subgroup of breast tumours. Although the experiments were not designed to answer the question about the origin of the somatostatin receptor expression, we found that somatostatin receptor expression indeed correlates with the size of the tumour and the absence of lymph node metastases but on the other hand may be an indicator of more aggressive disease. However, we found no specific morphological characteristics and furthermore observed heterogeneous somatostatin receptor expression in a significant subset of the tumours. These results are compatible with both possibilities. The absence of common genetic alterations might reflect that other, not investigated genetic alterations are involved or alternatively that different genetic alterations in the same pathway lead to either loss or gain of somatostatin receptor expression. Whatever the mechanism, to explain the

correlations with tumour characteristics and the course of disease, we have to assume that once expressed, the somatostatin receptor has a major influence on the biological behaviour of the tumour. This latter possibility underscores the importance of further functional investigations of the somatostatin receptor in breast cancer.

The molecular mechanism could be analogous to that described in several other human diseases, like hyperthyroidism and familial glucocorticoid deficiency. Mutations in G-protein coupled receptors have been shown to be the molecular defect in these traits. Interestingly, mutations have also been found in the coupled G-proteins in pituitary, thyroid, adrenal cortical and ovarian tumours.

These mutations might abolish or reduce the activity of the SSTR, indirectly enhancing cell growth in a tumour suppressor like way. The experiments described in chapter III.4 are the first attempt to study allelic imbalance of SSTR genes in breast cancer. Future research will be directed at the identification and characterization of more subtle alterations in the genes and the expression of somatostatin receptors in breast carcinomas.

It has been known for a long time that a family history of breast cancer increases the risk to develop a tumour, especially when first degree relatives are affected. From these data statistical risk estimates have been calculated and used to identify people at risk. The recent identification of genes and gene loci that are associated to breast cancer predisposition, marks a new era in this field. It enables more precise risk calculations, but can also identify the persons at risk within a family. This allows the persistent screening of the people who are really at risk and on the other hand relieves the family members that do not carry the predisposing gene from these screening procedures. Recently, in the Rotterdam and Leyden area working groups have been started where molecular and clinical geneticists and clinicians work close together to design a practical cancer prevention advice for family members at risk. It is our feeling that this collaborative approach will help to keep the physical and psychological burden of the families as low as possible and may also prove to be a cost-saving approach.

The identification of genes that predispose to familial breast cancer has also raised new questions that warrant further research. For instance: How is it possible that one gene can predispose to different familial breast cancer syndromes, like the putative *BRCA1* gene that is involved in both site specific familial breast cancer and in the breast-ovary cancer syndrome? And, how is it possible that only a few tissues transform to malignancy in response to the presence of the predisposing gene, while this gene is present in all somatic cells of the body? These questions show that only some aspects of breast cancer (and its heterogeneity) have been resolved and much remains to be learned from future research.

CHAPTER VII: APPENDIX

VII.1: ASSOCIATION BETWEEN ALTERATIONS OF THE *RB-1* TUMOUR SUPPRESSOR GENE AND FACTORS OF FAVOURABLE PROGNOSIS IN HUMAN PRIMARY BREAST CANCER, WITHOUT EFFECT ON SURVIVAL.

Submitted

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Abstract

The retinoblastoma (*RB*) tumour suppressor gene has not only been associated with retinoblastoma but also with several other tumours like osteosarcoma, small cell lungcarcinoma, prostate- and breast-cancer. We have studied the incidence of *RB* gene alterations in 96 primary breast tumours and in seven breast cancer cell-lines, using Southern blotting techniques. The outcome has been related with patient and tumour characteristics, oncogene amplifications, p53 mutations and with prognosis.

RB gene alterations were found to occur more frequently in estrogen receptor (ER) positive than in ER-negative tumours (26% *vs* 12%; n.s) and less frequently in tumours with oncogene amplification than in tumours without oncogene amplification of HER2/*neu*, c-*myc* or 11q13, (14% *vs* 31%; n.s.). *RB* gene alteration was observed both in tumours with (27%) and without a p53 gene mutation (22%). Data on 93 patients (mean age, 59.6 years; median follow up, 74 months) and *RB* gene alterations revealed a significant association between the frequency of *RB* gene alterations and node-negative patients ($p<0.01$) or smaller (<2 cm) tumours ($p<0.01$), but no relation with age, differentiation grade or (relapse-free) survival. Patients with and without RB gene alterations showed the same relapse-free and overall survival.

Introduction

The retinoblastoma (*RB*) susceptibility gene was the first gene identified as a tumour suppressor gene[1,2]. The *RB*-1 gene encompasses 27 separate exons distributed over about 200 kb of genomic DNA in the chromosome 13q14.2 region. It is ubiquitously expressed as a 4.7 kb mRNA transcript with a 2.7 kb coding region. The phosphoprotein product, $pp110^{RB1}$, is a nuclear protein that has DNA binding activity and plays a pivotal role in cell-growth control[3,4]. Survivors of retinoblastoma with germline mutations of the *RB*-1 gene are prone to develop osteosarcoma, soft-tissue sarcomas and other tumours later in life.

The observation that mothers of children with osteosarcoma or soft tissue sarcoma are at higher risk of developing breast cancer started analysis of chromosome 13q markers in human breast cancer[5].

Inactivation of tumour suppressor genes can result as a consequence of a combination of events including mutation, rearrangement or physical deletion of the gene. Loss of heterozygosity (LOH) of DNA sequences in the vicinity of the gene is considered indicative of suppressor gene inactivation. In human breast cancer allelic loss of the 13q arm was observed in about 25-35% of the cases studied[6-8]. The relationships between prognosis and oncogene amplifications or p53 gene mutations in human breast cancer have been studied considerably, but the relationship of *RB* gene alterations with respect to prognosis is still unclear. The study on the role of the *RB* gene (and its protein product) herein may be hampered by the several facts. Firstly, LOH of this gene is not always correlated with reduced expression of the pRB protein (in one report by Borg et al[9], even an increased expression has been described for cases with LOH). Secondly, LOH at the *RB* locus on chromosome 13 appears not to be related with physical deletion of the *RB* gene (as reported by Kalioniemi et al[10], who used an in situ hybridization technique with contiguous phage clones from the 3' and 5' ends of this gene). Therefore, in stead of testing LOH of 13 q, we have searched for deletions inner the *RB* gene with Southern blotting techniques, and have related *RB* gene alterations with oncogene amplification, p53 mutations, patient and tumour characteristics and with (relapse-free) survival.

Patients and methods

Patients: Frozen primary tumour tissue samples from 98 breast cancer patients, with no signs of distant metastasis at time of surgery, were used. Two patients were not evaluable for *RB* gene alterations. All these patients are part of a series of 282 patients studied for HER2/*neu* and c-*myc* amplification as described previously (Berns et al, 1992). Mean age of the patients was 59.6 years (range 28-84 year). Further patient and tumour characteristics are listed in Table I.

Sixty-six patients (71%) experienced a relapse and 53 patients have died during the follow up.

Cell-lines: In addition several cell-lines with different characteristics with respect to gene alterations were investigated. These mammary tumour cell-line include: EVSA-T, MCF-7, MDA-MB-231, SKBR-3, T47D, ZR-75, and ZRHerc, which is transfected with the EGF-R.

Assays for (onco)gene alterations and steroid hormone receptors: Tumour tissues and pellets of the breast tumour cell-lines and from human placenta or normal breast fibroblasts (negative control), were stored in liquid nitrogen. The samples were pulverized in the frozen state and homogenized in phosphate buffer according to the EORTC prcedure[12]. High molecular weight chromosomal DNA was isolated from an aliquot of the total homogenate and oncogene analysis was performed as described previously[11] using standard Southern blotting techniques with either EcoR1 digested DNA for the study of oncogene amplification (c-*myc*: a EcoR1-Cla1 human exon 3 specific c-*myc* probe; HER2/*neu*: pHER2-436-1, ATCC 59296; for 11q13: using the *int*-2 probe; SS6, a 0.9 kb Sac1-Sac1 fragment), or HindIII digested DNA for the study of *RB* gene alterations (probes pGH2 and pG3.8M, two EcoR1 cDNA fragments of 0.9 and 3.8 kb representing the 5′ and 3′ *RB* cDNA respectively, encompassing the entire coding region[1], as described previously[13].
Amplification of the oncogenes was defined as more than two copies of the (onco)gene studied, as examined with a Bio-Rad video densitometer 620. *RB* gene was defined as altered (AA) when both 3′ and 5′ hybridization patterns of the *RB* gene are changed by visual inspection of the blots. Mutations in the p53-gene were analyzed in exons 5 through 8 using PCR-SSCP techniques[14]. Cytosolic ER and PR levels were measured with ligand binding assay according to the EORTC procedures[12].

Statistics: Analysis of variance (ANOVA) and logistic regression analysis were used to study the associations between *RB* gene alterations and patient/tumour

TABLE I:Patient and tumour characteristics, oncogene amplifications, *P53* mutation and *RB* gene alterations (AA).

	Number of tumours[1]	*RB*-AA[2]	p-value
Total	*96 (100%)*	*24*	
Menopausal status			
Pre/peri	34 (37%)	26	
Post	59 (63%)	20	
Nodal status			
No	24 (26%)	46	0.01
N+	69 (74%)	14	
Tumour size			
T1	19 (21%)	53	0.01
T2	54 (58%)	15	
T3/4	19 (21%)	16	
Steroid receptor status			
ER+	68 (73%)	26	
ER-	25 (27%)	12	
PR+	62 (71%)	23	
PR-	25 (29%)	24	
Normal	45 (48%)[1]	31	
Amplified[3], (for any of 3 oncogenes)	49 (52%)	14	
c-*Myc*-normal	74 (77%)	24	
c-*Myc*-amplified[3]	22 (23%)	23	
HER2/*neu*-normal	76 (79%)	28	
HER2/*neu*-amplified[3]	20 (21%)	10	
11q13-normal[1]	80 (86%)	25	
11q13-amplified[3]	13 (14%)	8	
p53-normal	50 (62%)	22	
p53-mutated[4]	30 (38%)	27	

[1]Numbers do not always add up to 96, due to missing information for the subset studied.
[2]*RB* gene alteration, as described in the materials and methods section.
[3]Amplified, refers to more than two gene copy numbers. Numbers studied for amplification do not always add up to 96 since data on three samples were not evaluable for 11q13. Ten samples showed a co-amplification (HER2/11q13; HER2/c-*myc*; IGF1-R/11q13; c-*myc*/11q13)
[4]p53 mutations were analysed in 80 samples with PCR-SSCP of exons 5 through 8, as described in the materials and methods section.
$p < 0.01$: statistically significant.

characteristics and prognosis. Relapse-free and overall survival probabilities were calculated with the actuarial method of Kaplan and Meier[15]. Tests for differences or trends in (relapse-free) survival were performed with the likelihood ratio test in the univariate Cox regression model.

Results

Correlation between *RB* gene alterations and oncogene amplification or p53 gene mutation: Two samples of the 98 tumours studied, were not evaluable for *RB* gene alterations. Gross alterations in the *RB* gene were observed in 23 (24%) out of 96 evaluable human primary breast cancer samples studied (Table I). No *RB* gene alterations were observed in the mammary tumour cell-lines, EVSA-T, MCF-7, MDA-MB-231, SKBR-3, T47D, ZR-75 and ZRHerc studied, nor in mammary fibroblasts (not shown). Amplification of one or more oncogenes was observed in 49 (52%) of the primary tumours studied (Table I). c-*Myc* gene amplification was observed in 22 tumours (23%), HER2/*neu* gene amplification in 20 tumours (21%), IGF-1R gene amplification was observed in 4 out of the 96 tumours studied (not shown), while 11q13 amplification was observed in 13 (14%) of the 93 evaluable tumours. Ten tumours showed a co-amplification of the genes mentioned above. *RB* gene alterations were less prevalent in tumours with oncogene amplifications: 31% in tumours without vs 14% in tumours with gene amplification (Table I). When evaluated for separate oncogenes, *RB* gene alterations were less prevalent in tumours with a HER2/*neu* gene amplification (10% in tumours with HER2/*neu* amplification vs 28% in tumours without amplification) or with a 11q13 amplification (8% vs 25%). Interestingly, there appeared to be no difference in the incidence in *RB* gene alterations in tumours with or without an amplified c-*myc* gene (Table I). p53 mutations, as analyzed by PCR-SSCP on exons 5 through 8, were present in 38% (30 out of 80) breast tumour samples analyzed and in the cell-lines SKBR-3 (exon 5), T47D (exon 6), EVSA-T (exons 6&7) and MDA-MB-231 (exon 8). There is no difference in incidence of *RB* gene alterations between tumours with or without p53 gene

mutations (Table I).

Relationship of *RB* gene alteration with prognosis: Clinical data were available on 93 patients (Table I). *RB* gene alterations were most frequent in small tumours (T1; <2 cm) and in tumours from node-negative patients. These associations between *RB* gene alterations and tumour size or node-negativity were significant ($p=0.01$, Table I). In addition *RB* gene alterations were found to occur more often in ER-positive tumours, 26% as compared with 12% in ER-negative tumours, although this difference was not statistically significant. In spite of the association of *RB* gene alterations with these clinical and molecular biological factors associated with good prognosis, the relapse-free survival (at 5 years) was not different for patients with or without *RB* gene alterations (33% each; as shown in Table II and Figure 1, upper part). When stratified for *RB* gene alterations, we did observe a higher relapse-free survival rate (50%) at 5 years in those patients without amplification in their primary tumours in comparison with those patients with an oncogene amplification as shown in Table II (Peto logrank statistics: $p=0.07$, borderline significance), confirming our earlier results[11]. With respect to overall survival also no difference between patients with or without *RB* gene alterations in their primary tumours was observed (Table II and Figure 1, lower part). Using Cox multivariate regression analysis for RFS (including tumour size, nodal and receptor status, oncogene amplification and *RB* gene alterations) we observed that only nodal status and ER retained significance in this series of patients ($p=0.055$ and $p=0.001$, respectively).

TABLE II: (Relapse-free) survival of breast cancer patients with gene alterations in the primary tumours.

RB gene alterations	Patients #	RFS[1] 6 mo	OS [2] 0
Absent, in total	72*	33%	50%
Without gene-amplification*	30	43%	60%
With gene-amplification	41	24%	41%
Present, in total	21*	33%	49%
Without gene-amplification*	12	50%	62%
With gene-amplification	7	14%	43%

[1]RFS means relapse-free survival

[2]OS means overall survival.

*-11q13 amplification was not evaluable in three patients.

-Another three patients were not evaluable for RB gene alterations as related with clinical data.

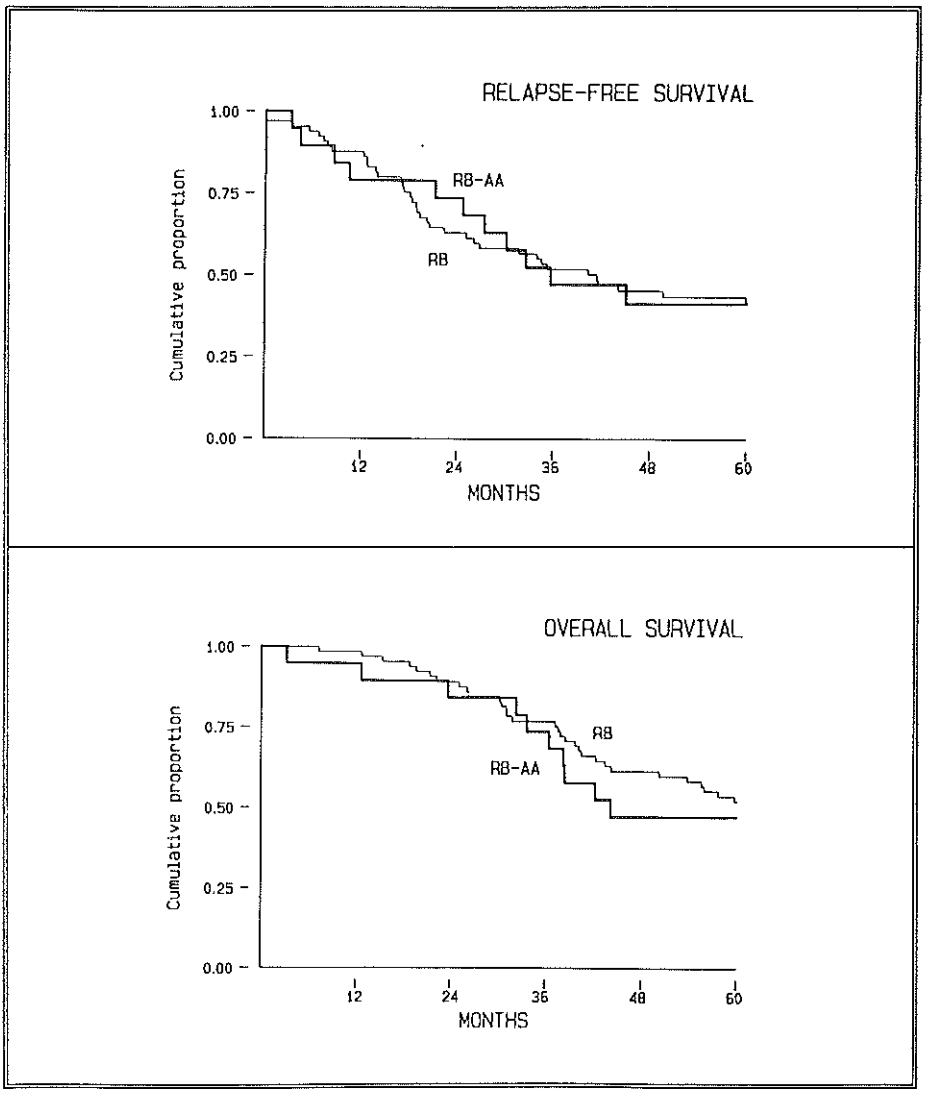

Figure 1: (Relapse-free) survival curves of patients with or without *RB* gene alterations

Upper panel shows relapse-free survival and lower panel shows overall survival as function of *RB* gene alterations for patients with (bold line: RB-AA) or without (thin line: RB) *RB* gene alterations.

AA means "Altered" and refers to gene alterations in the *RB* gene using both the 3' and the 5' *RB* probe.

Discussion

Successive loss of function of both alleles of the *RB* gene seems to predispose for development of retinoblastoma and osteosarcoma, but the relationship between the *RB* gene and the development of human breast cancer has yet to be clarified. The studies concerning *RB* gene in breast cancer are limited and the clarification of the role of the *RB*-1 gene in breast cancer genesis or prognosis has been hampered by the use of different techniques (e.g. molecular analyses studying either LOH of the 13q chromosome region or gross alterations in the *RB* gene and studies on protein expression using Western blotting or immunohistochemical techniques, all with different anti-RB monoclonal antibodies).

Using Southern blot analysis we observed structural alterations in 24% of the 96 tumours studied. This is in line with incidences of 19% (observed in 77 tumours) and 13% (in 79 tumours) published previously[13,16]. The data are also similar to findings obtained with LOH studies of several loci on 13q (including *RB*) since LOH was observed in 25-35% of the primary breast cancers studied[6-9,17].

With respect to tumour and patient characteristics, we observed that *RB* gene alterations, as studied in 96 tumours, are significantly more frequently found in tumours of node-negative patients and in smaller (T1) tumours. Moreover *RB* gene alterations were found more often in ER-positive tumours and in tumours without oncogene amplifications, all indicative of tumours with a relative good prognosis. Thorlacius et al[17], did not observe a relation with different clinical parameters, including tumour class, nodal status, stage or receptor status, but they observed that abnormalities at the *RB*-1 locus and abnormalities on chromosome 17 frequently occurred simultaneously. In contrast to our investigations of *RB* gene alterations, Varley et al[16] and Borg et al[9] did show a relation between LOH of the *RB* gene and aggressiveness of tumours and poor prognosis. Borg et al[9] observed significant correlations between *RB* gene alteration and DNA nondiploidy, high S-phase fraction and LOH on chromosome 17p13.3. In addition, they observed no relation with tumour size and lymph

node involvement (90 informative patients), and no correlation between LOH of the *RB* gene and ER or PR status or oncogene amplification[9]. However the loss of pRB expression was inversely related to HER2/neu amplification. Moreover, Varley et al, observed that the incidence in the genetic changes of the *RB* gene increased in tumours with poorer differentiation and with the degree of tumour spread[16]. Using immunohistochemical techniques Trudel et al[18], who studied 100 breast cancers, observed a correlation between the presence of pRB and grade 3 tumours, whereas absence of pRB was correlated with increased p53 expression. In their study there was no discernible correlation of *RB* expression with other clinical parameters[18].

With respect to the relationship of *RB* gene alterations and (disease-free) survival in breast cancer data of only few studies are available. We did not observe an association between alterations in the *RB* tumour suppressor gene (*RB*-AA) and the lenght of disease-free or overall survival. Sawan et al, also observed no relationship between pRB expression and relapse or survival in 197 patients studied but, in contrast to our data, they observed a significant correlation with positive lymph node status[19].

From these contrasting data, it is difficult to draw specific conclusions about the role of the *RB* gene in human primary breast cancer. From a methodological point of view, criticism on the study of the *RB*-1 gene in breast cancer is warranted since *RB*-1 allele loss (LOH) was not always associated with the loss of pRB protein expression. In addition LOH of loci on chromosome 13 (including the *RB* locus) appeared not to be related with physical deletion of the *RB* gene. This may imply that another gene (or genes) located on chromosome 13q could be related with the progression of most human breast cancer patients. Particularly relevant to this possibility is the recent report by Schott et al[20] who isolated a gene proximal to the *RB-1* gene (located at 13q12-q13), named *Brush-1*. They were able to show that LOH of this gene is related with decreased *Brush* mRNA levels whereas *RB-1* mRNA levels remained unchanged. Although *Brush-1* is a likely candidate it needs further study since tumour and patient data, which allow for interpretation of clinical relevance, are as yet unavailable for the *Brush-1* gene.

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CHAPTER VIII: SUMMARY

Breast cancer is a frequent disease that affects approximately one out of twelve women in the western world. It is also a capricious disease with a mild course in one patient while another patient can be attacked by a very aggressive type of breast cancer. Heterogeneity is been observed at the cellular level. Genetic changes accumulate and eventually change a normal breast cell into a malignant one. It has been shown that in different breast tumours, different combinations of genetic alterations are present. These changes affect oncogenes and tumour suppressor genes, genes that in the normal somatic cells are involved in cell growth and differentiation. With the experiments described in this thesis the relation between these genetic changes, the phenotype of the tumour and the course of the disease was investigated. To this end we delineated the heterogeneity of breast cancer through the selection of three homogeneous subgroups of breast tumours.

In chapter III.1 and III.2 expression of the somatostatin receptor is used as a selection criterion. In chapter III.1 the association of the expression of this receptor with other tumour and patient characteristics was investigated. Furthermore, subgroup specific genetic alterations were sought. We observed that somatostatin receptor expression is common, especially in smaller tumours. No indications of subgroup specific genetic alterations were detected. In chapter III.2 we report upon the results of a study on the incidence of somatostatin receptors in breast cancer using *in-vivo* scanning with a radiolabeled somatostatin analog as a probe. A similar high incidence was found using *in-vivo* scanning as was obtained using *in-vitro* autoradiography. Moreover, somatostatin scintigraphy turns out to be a very sensitive tool in the early detection of distant metastases. The clinical relevance of this early detection remains to be determined.

The peptide hormone somatostatin has growth inhibiting potential both *in-vitro* and *in-vivo* in animal model systems and pituitary tumours. This process is mediated by binding of the hormone to its receptor. Loss of function of the somatostatin receptor could in theory contribute to enhanced cell growth and

might thus lead to tumour progression. Change of function of the somatostatin receptor can be the result of changes in the somatostatin receptor protein levels, which in turn can be influenced by alterations in the somatostatin receptor genes. Five somatostatin receptor genes have been isolated recently. Of two of these receptors (SSTR1 and SSTR2) the chromosomal localization and the copy number of the gene in primary breast cancer were investigated (chapter III.4). The SSTR1 gene has been mapped to chromosome 14q13 and showed allelic imbalance in 34 of 67 informative tumours. The SSTR2 gene is at chromosome 17q24 on the long arm of chromosome 17. Allelic imbalance was observed in 40 of 77 informative tumours. This suggests that the SSTR2 is involved in the genetic changes of chromosome 17q that have been observed in breast cancer.

In chapter IV.1 and VII, tumours that contain alterations in the retinoblastoma tumour suppressor gene were investigated. We observed a correlation between the presence of these alterations and a small size of the tumour and also with an absence of lymph node metastases in the patient. Both characteristics are markers of a better prognosis and therefore we expected that also the presence of *RB* gene alterations would be indicative of a favourable prognosis of the patient. However, we did not find a correlation between the presence of *RB* gene alterations with the duration of the disease free interval or with overall survival (chapter VII).

The third approach was the analysis of breast cancer patients that derive from families with a hereditary predisposition to breast cancer. These people share a common predisposing genetic marker. In chapter IV.2 the contribution of the Medical Genetics Centre in a European effort to identify one breast cancer predisposition gene, the *BRCA1* gene is described. This *BRCA1* gene is involved in approximately 40% of families that express breast cancer with an age at onset under 47 years, and also in most hereditary, breast-ovary cancer families. Using linkage analysis, the *BRCA1* gene has been mapped to chromosome 17q21. Now many research groups are trying to identify and clone the gene using different molecular biological techniques.

In chapter V the results of an investigation of functional changes in

nonmalignant cells from members of a 17q-linked breast cancer family are reported. Earlier research in several familial cancer syndromes showed an enhanced induction of the so-called SOS DNA repair mechanism. Although the mechanism of this enhanced induction is at present unknown, the phenomenon of enhanced induction has been correlated with the expression of cancer in these families. The 17q-linked breast cancer family under investigation in chapter V.1 shows enhanced induction of the SOS-repair system to moderate levels. In contrast to for instance the results obtained from Li-Fraumeni families, no relation with disease expression was detected.

In conclusion, delineation of breast cancer heterogeneity through the selection of specific subgroups and the identification of molecular changes that play a role in these tumours, helps the correlation of these changes to the phenotype of the tumour and to the course of the patients' disease. It will take much more research before this fundamental knowledge will be applied in clinical practice.

CHAPTER IX: SAMENVATTING

Borstkanker is een frequent voorkomende aandoening die ongeveer 1 op de 12 vrouwen treft. Het is ook een ziekte met vele gedaantes, die bij de ene patiënt een mild beloop toont, terwijl een volgende patiënt wordt overvallen door een zeer agressieve aandoening. Dit heterogene karakter wordt ook op cellulair niveau waargenomen. Op DNA niveau vinden genetische veranderingen plaats, die van een gewone borstkliercel een kankercel maken. Het blijkt dat in verschillende tumoren combinaties van verschillende genetische veranderingen kunnen worden gevonden. Het betreft veranderingen in oncogenen en tumorsuppressiegenen, genen die in een gezonde lichaamscel een rol spelen bij de groei en differentiatie van die cellen. Het werk beschreven in dit proefschrift wil door middel van het ontrafelen van de heterogeniteit van borstkanker onderzoeken of er een relatie bestaat tussen de veranderingen op genetisch niveau met de uiterlijke kenmerken van een borsttumor of het beloop van de ziekte. Daartoe zijn series borsttumoren en borstkankerpatiënten op een drietal wijzen geselecteerd. In paragraaf III.1 en III.2 wordt expressie van de somatostatine receptor als uitgangspunt gekozen. In paragraaf III.1 wordt de relatie tussen de aanwezigheid van de receptor op de tumor met andere tumorkenmerken, waaronder specifieke genetische veranderingen, onderzocht. Het blijkt dat expressie van de somatostatine receptor frequent voorkomt, met name in kleinere tumoren. Aanwijzingen dat in de etiologie van deze groep tumoren een kenmerkende genetische verandering ten grondslag ligt werden niet gevonden. In paragraaf III.2 worden de resultaten van de detectie van somatostatine receptoren door middel van scintigrafie van patiënten met behulp van een radioactief somatostatine analoog besproken. Ook uit deze studie blijkt somatostatine expressie frequent in borstkanker detecteerbaar zijn. Bovendien blijkt somatostatine scintigrafie een gevoeliger methode voor vroegdetectie van uitzaaiingen te zijn dan de thans gebruikte methoden. De klinische betekenis van deze vroegere detectie is nog niet bekend. De tumoren die gebruikt werden in III.1 werden voor een deel verkregen na operatie van de in III.2 onderzochte patiënten.

Het eiwit hormoon somatostatine heeft zowel in celkweek experimenten als in diermodellen en menselijke hypofysetumoren celgroeiremmende eigenschappen. Dit effect komt tot stand door binding van het hormoon aan de somatostatine receptor. Functie vermindering of -verlies van de somatostatine receptor zou theoretisch tot versnelde celgroei kunnen leiden en op die manier bijdragen aan de groei van een borsttumor. Deze veranderde functie kan veroorzaakt worden door veranderingen in het gen dat codeert voor de somatostatine receptor. Er zijn vijf van dergelijke genen geïsoleerd. Van twee daarvan (SSTR1 en SSTR2) wordt in paragraaf III.4 de chromosomale lokalisatie en het aantal genkopiën in borstkankercellen beschreven. Het SSTR1-gen ligt op de lange arm van chromosoom 14 (14q13) en toonde kwantitatieve veranderingen in 23 van 67 onderzochte en beoordeelbare tumoren. Het SSTR2 gen ligt op de lange arm van chromosoom 17 (17q24) en is veranderd in 40 van de 77 informatieve tumoren. Dit suggereert dat veranderingen in dit somatatinereceptorgen onderdeel zijn van de genetische veranderingen van de lange arm van chromosoom 17 in borstkanker.
In paragraaf IV.1 en VII werden tumoren met veranderingen in het retinoblastoma tumorsuppressiegen bestudeerd. De aanwezigheid van deze veranderingen blijkt te correleren met een klein formaat van de tumor en de afwezigheid van lymfekliermetastasen in de patiënt, waardoor de verwachting was dat veranderingen in het retinoblastoma gen een aanwijzing zouden zijn voor een gunstige prognose voor de patiënt. In hoofdstuk VII blijkt echter dat veranderingen in het retinoblastoma tumorsuppressiegen geen effect hebben op de lengte van de ziekte vrije periode na de eerste behandeling en ook niet op de uiteindelijke overlevingsduur van de patiënten.

De derde groep borstkanker patiënten komt uit families waarin erfelijke aanleg tot het ontstaan van borstkanker voorkomt. In paragraaf IV.2 wordt de bijdrage van de werkgroep mammatumoren van het Medisch Genetisch Centrum beschreven aan het Europese onderzoek dat tot doel heeft het *BRCA1* gen te identificeren. Dit *BRCA1* gen veroorzaakt de aanleg tot het krijgen van borstkanker in families waarin de borstkanker voor het 47ste levensjaar tot

uiting komt, en in families met aanleg tot het ontwikkelen van zowel borst- als eierstokkanker. Door middel van koppelingsonderzoek, waaraan het in IV.2 genoemde werk een bijdrage is, bleek dat het *BRCA1* gen ligt op de lange arm van chromosoom 17 (17q21). Met behulp van meer gedetailleerd koppelingsonderzoek en andere moleculair biologische technieken wordt thans getracht het gen te kloneren. Paragraaf V.1 beschrijft een studie naar een functionele verandering in niet kwaadaardige cellen van individuen uit een familie met erfelijke aanleg voor borstkanker. Uit eerder onderzoek was komen vast te staan dat in een aantal familiaire kanker syndromen een verhoogde induceerbaarheid wordt gevonden van een DNA herstel mechanisme, het zogenaamde SOS herstelmechanisme. Deze verhoogde induceerbaarheid is geassocieerd met het optreden van de kanker in deze families. Het mechanisme dat hieraan ten grondslag ligt is nog onbekend. In paragraaf V.1 wordt onderzoek van dit SOS herstelmechanisme in een aan chromosoom 17q gekoppelde borstkanker familie beschreven. Ook hier wordt een verhoogde induceerbaarheid van dit DNA herstel mechanisme gevonden. Doordat dit zowel het geval is bij familieleden met borstkanker als gezonde familieleden, kon een directe relatie met het optreden van borstkanker in deze familie niet worden aangetoond. Dit in tegenstelling tot hetgeen gevonden werd in een andere vorm van erfelijke borstkanker, het Li-Fraumeni syndroom.

Het in dit proefschrift beschreven onderzoek laat zien dat door het ontrafelen van de heterogeniteit van borstkanker het inderdaad mogelijk is verbanden te leggen tussen de moleculaire veranderingen die leiden tot borstkanker en het ziektebeloop van de patiënt. Veel onderzoek zal echter nog nodig zijn voordat deze fundamentele kennis directe gevolgen gaat krijgen voor de patiënt en haar familie.

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jan 1992	Aanvang specialisatie Inwendige Geneeskunde op de afdeling Inwendige Geneeskunde III van het Academisch Ziekenhuis Rotterdam Dijkzigt (opleider: Prof. Dr. J.C. Birkenhäger).

NAWOORD

Dit proefschrift werd bewerkt onder leiding van prof dr SWJ Lamberts (afdeling Inwendige Geneeskunde III, tevens Endocrinologie en Stofwisselingsziekten) en dr JEMM de Klein (afdeling Celbiologie en Genetica). Het praktische werk werd, als gast van de afdeling Pathologische Anatomie, gedaan in nauwe samenwerking met de groep van dr EC Zwarthoff. Verder konden vruchten worden geplukt van de leerzame en produktieve samenwerking binnen de werkgroep mammatumoren van het Medisch Genetisch centrum Zuidwest Nederland.
Aan het eind gekomen van dit proefschrift wil ik dank betuigen aan al diegenen die mij met raad en daad hebben bijgestaan om in twee sterk verschillende werelden overeind te blijven; enkelen van hen wil ik met name noemen:
Mijn promotor en co-promotor, die mijn doen en laten de afgelopen jaren nauwgezet en geduldig gevolgd en gestuurd hebben.
Ellen, Ronald, Peter, Arnold, Nick, Karin, Nicole en Renée van Pel met wie bij de PA werd samengewerkt. Dirk, Anne, Ellen en Hanneke van de afdeling Celbiologie en Genetica. De Leidenaren Cees Cornelisse, Péter Devilee en Peter Abrahams en uit de Daniël Jan Klijn, Els Berns en John Foekens. En "Last but not least" Casper van Eijck, Leo Hofland en Jean Claude Reubi. Jullie allen dank ik in het bijzonder voor de leuke en stimulerende samenwerking. De staf, collega assistenten, de verpleging en de polidames van interne III voor de belangstelling en het begrip dat ook het maken van dit "schriftje" tijd vergt.