Next Generation Diagnostic Molecular Pathology

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Next Generation Diagnostic Molecular Pathology

‘Next generation’ moleculaire diagnostiek in de pathologie

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General introduction

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Pathology is the medical discipline that diagnoses disease by investigation of tissues, cells and bodily fluids. The pathology diagnosis forms the basis of many medical treatments. Generally, tissue specimens obtained from a patient by small biopsies are formalin fixed in its entirety prior to further processing in tissue blocks, except when additional techniques require native material. In case of larger resection or excision specimens, preliminary examination and description by a pathologist is required, while preserving fresh samples for biobanking prior to formalin fixation. After fixation, further sampling of resection specimens ('grossing') is performed in order to select tissue parts in small cassettes for microscopic evaluation. The selected formalin fixed tissue parts are subsequently embedded in paraffin. The formalin fixed and paraffin embedded (FFPE) tissue blocks are sectioned on a microtome and the tissue sections of a few micrometers thick are glued on glass slides. After deparaffinization the sections are generally stained with hematoxylin and eosin (H&E) to obtain cellular and nuclear detail, which is evaluated by microscopic examination (histopathology). If needed, additional information can be obtained from the tissue sections by immunohistochemical stainings, which visualize the presence or absence of specific cellular or extracellular constituents. For tumor evaluation, traditional histopathology and immunohistochemistry is more and more complemented with molecular pathology (figure 1). Diagnostic molecular pathology aims at facilitating proper diagnosis, prognosis and/or treatment of patients with cancer or suspected cancer by analyzing aberrations in the nucleic acids DNA and RNA. This thesis will focus on DNA analysis.

DNA (deoxyribonucleic acid) is a complex molecule that contains the information needed for development, functioning and reproduction of living organisms. Four different bases (adenine, thymine, guanine and cytosine) code this information; these bases are attached to a sugar-phosphate backbone, together called a nucleotide. Usually, DNA is organized as a double helix formed by two long strands of DNA, with bases from both strands joined as base pairs. Human DNA is organized into 23 chromosome pairs (one set inherited from each of the parents) that are located in the cell nucleus. Additionally a small

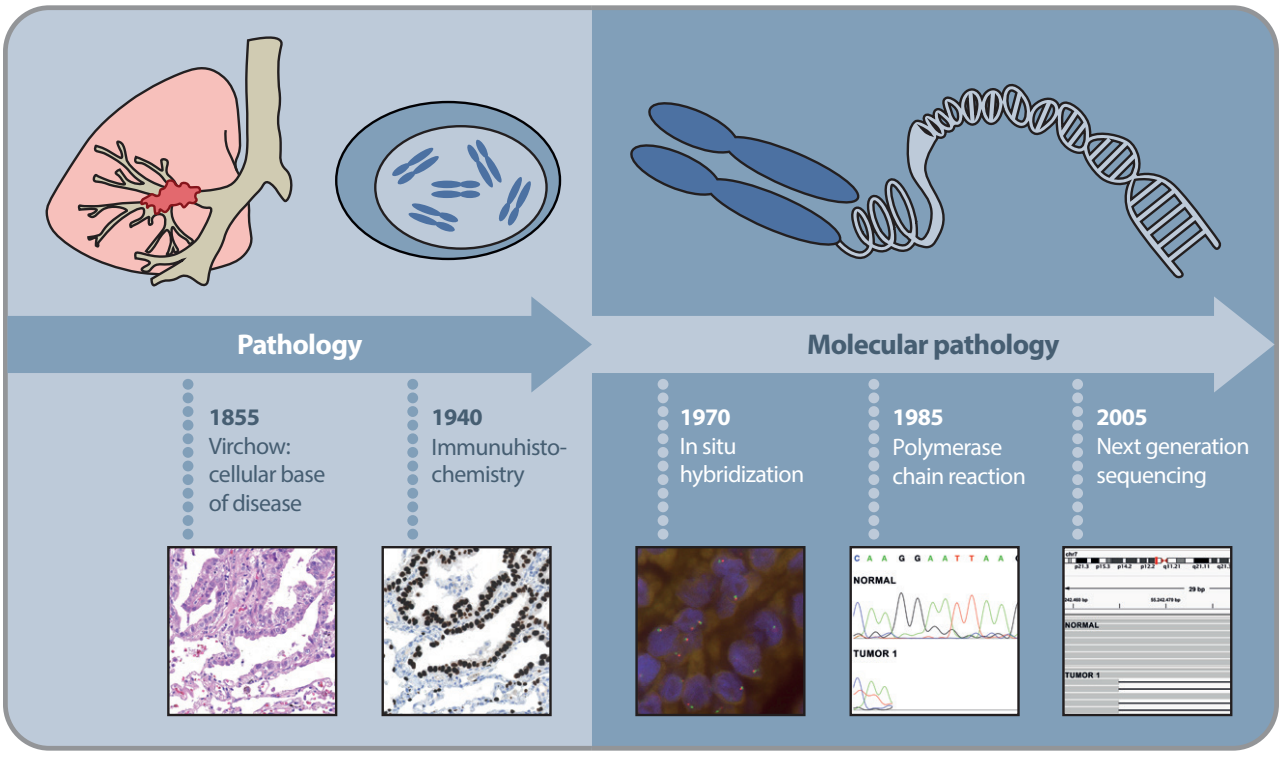


Figure 1. Molecular pathology timeline.

amount of DNA is present in the mitochondria. Mitochondrial DNA (mtDNA) is circular, present in numerous copies per cell and is inherited solely from the mother.

The general human DNA sequence can be described by approximately 3 billion base pairs; this sequence is largely identical for all human beings. However, approximately 0.5% of the DNA sequence differs between two human individuals. Most of these variations are neutral, which means that they have no selective effect on the organism. The most common type of sequence variation is the single nucleotide polymorphism (SNP), which is a difference of one base in the DNA sequence that occurs in at least 1% of the population. DNA profiling (to distinguish one individual from another) is usually based on short tandem repeat (STR) analysis. STR are repetitions of short sequences of nucleotides with variable lengths that are present on many chromosomes. Multiple other types of genetic variation exist, including copy number variations and epigenetic variations.

Cancers arise due to mutations in the DNA, which in contrast to neutral variants have a pathogenic effect. Tumor cells accumulate mutations as a result of genomic instability, with chromosomal and microsatellite instability as the most common mechanisms (figures 2 and 3). Chromosomal instability (CIN) is detected in the majority of tumors, and involves deletions or gains of whole chromosomes or parts of chromosomes. Microsatellite instability (MSI) is a type of genetic hypermutability, which is most pronounced in repeated sequences of DNA, the microsatellites. Other mechanisms resulting in DNA mutations include exposure to mutagens (smoking or ultraviolet light) and abnormal activity of enzymes that modify DNA (APOBEC) or of error-prone polymerases (POLE)[1]. During tumorigenesis, genomic instability mechanisms are considered to result in the activation of proto-oncogenes and the inactivation of tumor suppressor genes, leading to the transformation of a normal cell into a tumor cell. The mutated proto-oncogenes and tumor suppressor genes are drivers of the tumor and render oncogene addiction

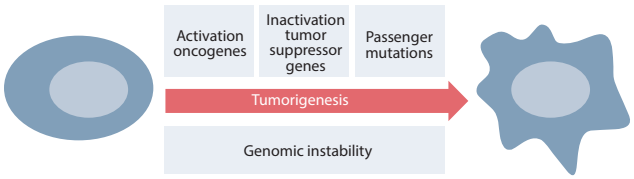


Figure 2. Tumorigenesis.

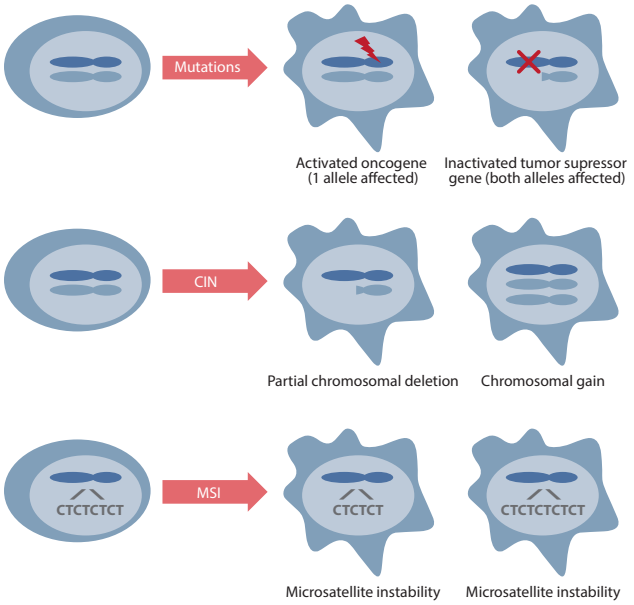


Figure 3. Chromosomal or genetic changes due to mutations, chromosomal instability (CIN) and microsatellite instability (MSI).

to the tumor cells. In addition to driver mutations, genomic instability will result in the occurrence of mutations without effect on the phenotype, the so-called passenger or hitchhiker mutations.

Most tumors are the result of somatic mutations, these mutations occur sporadically in any cell of the body. However, a minority of cancers arises in the context of a hereditary cancer syndrome, a disorder in which mutations are inherited from one of the parents. These inherited mutations are called germline mutations and are usually present in all cells of the affected individual. Most hereditary cancer syndromes are caused by germline mutations in tumor suppressor genes, which need a second hit before the gene is inactivated[2] (figure 4). The presence of a germline mutation (the first hit) predisposes the affected individual to cancer development.

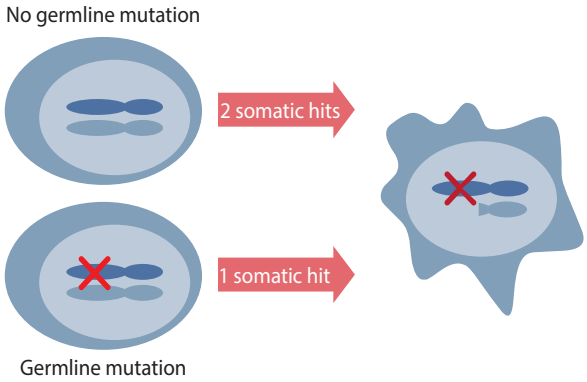
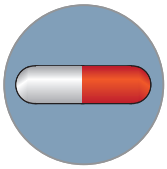


Figure 4. Inactivation of a tumor suppressor gene with and without the presence of a germline mutation.

Diagnostic molecular pathology

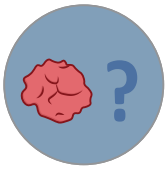
An overview of the routine molecular tests performed at the Erasmus MC department of pathology is shown in Table 1. The main reasons for testing for somatic DNA aberrations are:

To stratify patients for (targeted) therapy



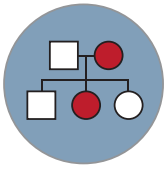
For some tumor types, the optimal treatment depends on the presence or absence of specific somatic DNA aberrations. Targeted therapies, for example, attack the tumor cells by specifically blocking mutated proteins or essential pathways that drive cell growth and proliferation. Molecular analysis reveals the driver mutations of tumors, and is therefore essential to stratify patients for the appropriate targeted therapy.

Differential diagnosis



Analysis of somatic DNA aberrations is also useful for differential diagnosis, to confirm or exclude a particular diagnosis. Some tumor types are characterized by the presence of certain genetic aberrations, for example a specific mutation in *MYD88* is detected in up to 90% of Waldenström macroglobulinemia (WM)[3]. As this mutation is highly specific for WM, identification of a *MYD88* mutation can help in diagnosing WM if histopathological evaluation is not conclusive.

In the context of hereditary testing



Detection of somatic DNA aberrations in tumor cells can help in deciding whether or not a patient should be referred for germline testing, as some hereditary syndromes are characterized by specific somatic aberrations.

In the last five years, molecular pathology has become increasingly important in patient care. At the Erasmus MC, this is illustrated by the transition of the laboratory from semi-research to ISO certified. Furthermore, molecular results are no longer hidden in the 'notepad' of the laboratory information management system, but are now a prominent part of the pathology report. In 2013 an official 2-year educational program for clinical scientist in molecular pathology (Klinisch moleculair bioloog in de pathologie) was established in the Netherlands. From 2014, all laboratories performing molecular testing in the Netherlands are required to employ a Clinical Scientist in Molecular Pathology, or at least have access to their expertise, as was described in guidelines introduced by the Dutch Society of Pathology (Nederlandse Vereniging voor Pathologie).

Molecular pathology is a quickly evolving discipline: implementation of new research discoveries as well as new techniques into the routine diagnostic setting is constantly performed. Targeted treatment options increased rapidly the last several years for multiple tumor types, including lung adenocarcinoma, colorectal carcinoma and melanoma[4-6]. Additionally, multiple resistance mechanisms to these targeted treatments have been described, part of which are targets for treatment itself[7,8]. To detect all aberrations relevant for a specific tumor type, molecular tests need to be adapted continuously.

One of the most important changes in diagnostic molecular pathology over the past few years was the introduction of next generation sequencing (NGS)[9-11]. Multiple different platforms for NGS are currently available, of which the Ion Torrent

Table 1. Overview of routine molecular tests performed at the Erasmus MC department of Pathology, Rotterdam (excluding in situ hybridization). Only the clinically most relevant genes are shown.

Tumor type	Molecular abberations tested	Reason for testing		
		Therapy	Differential diagnosis	In the context of heriditary testing
Brain tumors	*IDH1/2, ATRX, TP53, EGFR, PTEN, TERT, CIC, FUBP1* mutations & copy number aberrations of chromosomes 1p, 7, 9, 10, 12 and 19q	v	v	
	MGMT methylation	v		
Colorectal carcinoma	*KRAS*, *BRAF*, *NRAS* mutations	v		
	MSI	v		v
	MLH1 promoter methylation			v
	MMR gene mutations			v
Desmoid tumors	*CTNNB1* mutations		v	
Gastro-intestinal stromal tumors	*KIT*, *PDGFRA*, *BRAF* mutations	v	v	
	SDH gene mutations			v
Langerhans cell histiocytosis	*BRAF* mutations		v	
Melanoma	*BRAF*, *NRAS*, *KIT, GNAQ, GNA11* mutations	v		
Non-small cell lung carcinoma	*EGFR, KRAS*, *HER2*, *BRAF, MET* mutations	v		
Ovarian carcinoma	MSI, *MLH1* promoter methylation			v
	MMR gene mutations			v
	FOXL2 mutations		v	
	BRCA1/2 mutations	v		v
Pheochromocytoma, paraganglioma	SDH gene*, RET, VHL, NF1* mutations			v
Thyroid carcinoma	*RET, KRAS, NRAS, HRAS, BRAF* mutations		v	
Waldenström macroglobulinemia	*MYD88* mutations	v	v	
Multiple tumor types: clonality determinations	Mutations & copy number aberrations in multiple genes		v	
Multiple tumor types: tissue identification	Short tandem repeat analysis		v	

PGM (Life) and the MiSeq (Illumina) are predominantly used in molecular pathology laboratories in the Netherlands. Most diagnostic laboratories use *targeted* NGS, meaning that only a subset of genomic regions is screened, rather than the whole genome or exome. The basic principle of NGS is that, in contrast to Sanger sequencing, numerous small DNA fragments are amplified simultaneously. From each DNA fragment, hundreds to thousands of individual molecules are sequenced ('massive parallel') (figure 5). Therefore, it is possible to analyze a multitude of genes even when only a limited amount of tissue is available, as often is the case in diagnostic molecular pathology. Because every single molecule results in a separate read (DNA sequence), NGS is more sensitive in mutation detection compared to Sanger sequencing.

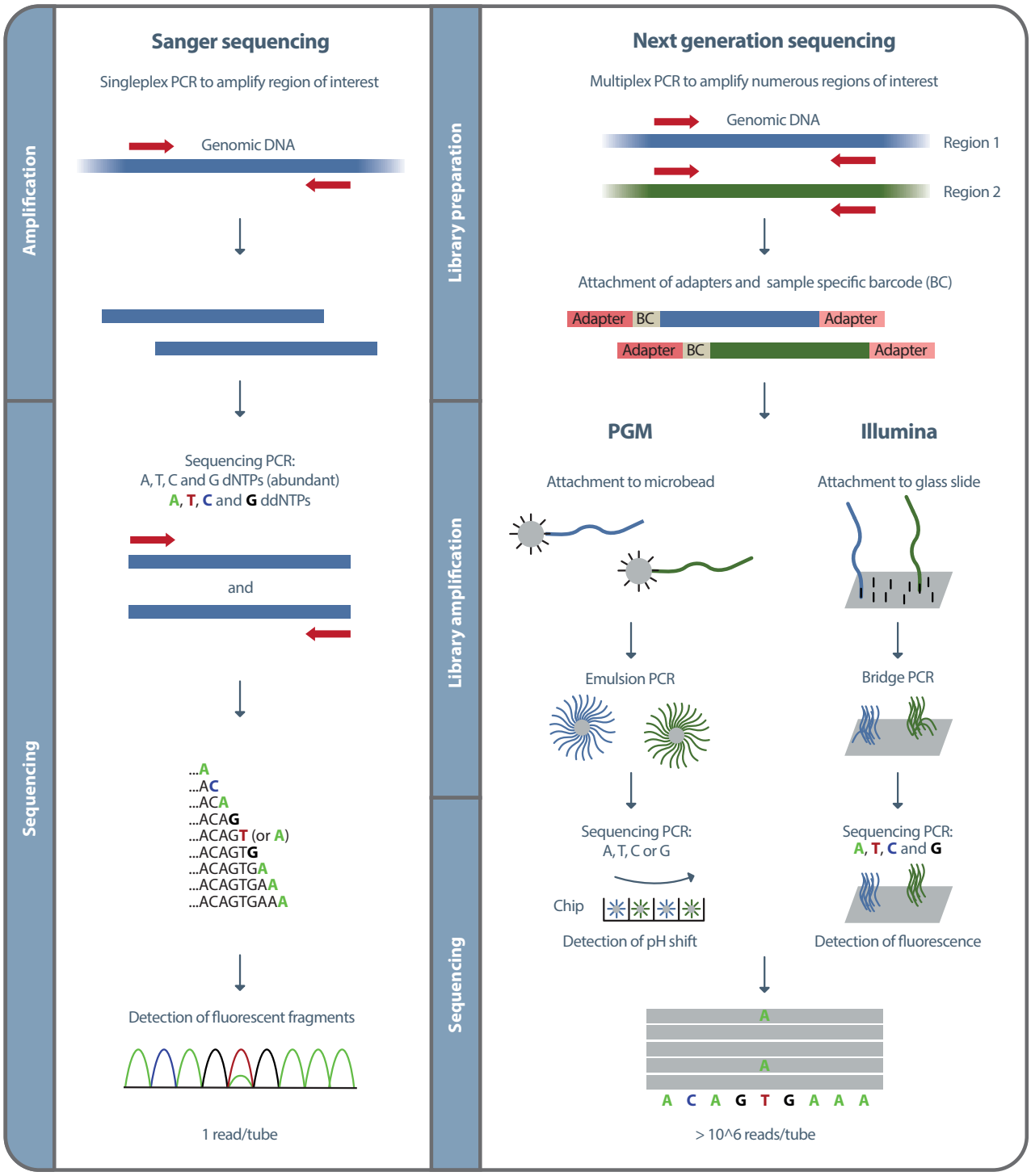
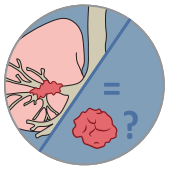


Figure 5. Sanger sequencing versus targeted next generation sequencing.

Additionally, NGS can also be used for copy number detection, for example using SNPs. Informative SNPs, which are sequence variants that are present on one allele, but not on the other, can be used to discriminate between both alleles. If no copy number aberrations are present, all informative SNPs should be present in a 1:1 ratio (equal amount of both alleles). If however loss or gain of a chromosomal region is present, the ratio will diverge from 1:1 indicating a copy number change. Another way to detect copy number aberrations is using coverage analysis. The coverage of a DNA fragment represents the total number of reads that is detected for that fragment. NGS is based on a multiplex PCR, during which some DNA fragments amplify more efficient than others. The coverage of a specific DNA fragment relative to the total number of reads for that sample, is however quite stable. A high or low relative coverage therefore implies that there is gain or loss, respectively, of that particular chromosomal region.

Clonality determinations



Some patients present with multiple tumors, either synchronous (at the same time) of metachronous (with time in between). These tumors can either be multiple primary tumors, or can be one primary tumor with one or more metastasis (metastatic disease). It is of prime importance to distinguish between these possibilities for patient care[12], however, this is not always possible based on clinical and histopathological characteristics.

Each primary tumor has particular unique somatic genomic aberrations, and detection of these aberrations can facilitate proper diagnosis for these patients. If multiple tumors show similar DNA aberrations this suggest that they have a common origin, whereas different aberrations suggests multiple origins. An ideal clonality marker gives unique results for every single tumor tested (high predictive power). This is not limited to somatic aberrations, but can also be other unique characteristics of a cell, as long as they are helpful in discriminating one tumor from another.

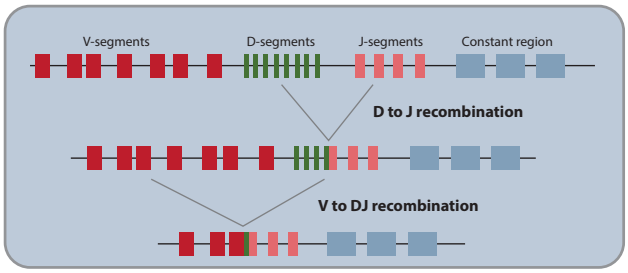
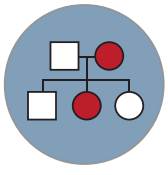


Figure 6. V(D)J recombination during lymphocytic development.

Examples of unique markers are the highly diverse immunoglobulin gene rearrangements in B-cells. During lymphocytic development, a mechanism called V(D)J recombination occurs, which is the random rearrangement of variable (V), diversity (D) and joining (J) gene segments (figure 6). This mechanism results in a highly diverse repertoire of immunoglobulins for antigen detection. As B-cell lymphoma are clonal expansions of one particular B-cell, all lymphoma cells harbor the same V(D)J rearrangement, which is highly unique for that particular tumor.

Unfortunately, for most tumors unique markers are not available. For most clonality determinations in molecular pathology, tumors are therefore broadly screened for mutations as well as copy number aberrations. Genes frequently affected in a wide range of tumor types are for example oncogenes of the mitogen-activated protein kinase (MAPK) or PIK3CA pathway and tumor suppressor genes like *TP53*, *PTEN* and *CDKN2a*. Additionally, many tumors show loss of heterozygosity (LOH) of (part of) chromosomes. The predictive power of these aberrations for determining tumor clonality varies, as some aberrations are more unique than others, also depending on the tumor type in which they are detected. However, testing a combination of multiple markers, each of low predictive value, can also help in determining tumor clonality.

Molecular pathology in the context of hereditary testing



Some tumors arise in the context of a hereditary syndrome; mutations in the germline can predispose for specific tumor types. In the

Netherlands, patients suspected of being germline mutation carriers are counseled by the department of clinical genetics. This suspection can for example be based on a young age at diagnosis, the presence of multiple tumors, or family history. Some hereditary syndromes are characterized by specific somatic aberrations, as is the case for Lynch syndrome (LS). Patients with LS have germline mutations in one of the mismatch repair (MMR) genes or a germline deletion in *EPCAM*, which predispose for various types of cancer, including colorectal and endometrial cancer[13-16]. Tumors of LS patients have common somatic aberrations: MSI and aberrant MMR protein expression. If a patient is suspected of LS, screening of the tumor(s) for MSI and MMR protein expression helps to decide whether or not the patient should be referred for counseling[17].

Additionally, testing for somatic aberrations can also complement hereditary testing during counseling. For tumor suppressor genes, germline mutations usually are only the first hit, and a second hit is needed to inactivate the gene and to drive tumorigenesis. This second hit is mostly loss of the wildtype allele (figure 4), which can be detected by somatic analysis of the tumor. When a germline variant of unknown significance is found in a patient suspected of a hereditary syndrome, somatic analysis can help in determining whether this variant might be pathogenic. For some patients suspected of a hereditary syndrome, no germline mutations or variants are detected in the specific genes known to be implicated in that particular syndrome. Detection of somatic aberrations in these genes suggests that these tumors have a sporadic origin and therefore facilitates proper diagnosis of these patients and their relatives[18,19].

Outline of the thesis

In this thesis studies are described on the application of a variety of molecular analyses in solving diagnostic difficulties that remained unsolved based on clinical and histopathological characteristics. The first part, clonality determinations, focuses on the use of unique DNA markers or somatic DNA aberrations to define tumor origin or the clonal relationships between multiple tumors from one patient. The second part, molecular diagnostics in the context of hereditary testing, focuses on the interface of germline and somatic diagnostics by clinical genetics and pathology, respectively. The central theme of this thesis is the application of molecular analyses in a routine diagnostic setting.

Part I - Clonality determinations

In **chapter 2** a patient with a metastasis of a colorectal carcinoma in a transplanted liver is described. As a colonoscopy was negative for this patient, the diagnostic question was raised whether this tumor originated from the patient itself or from the donor. As somatic aberrations of the tumor are not helpful for this question, unique patient specific markers were tested to determine the origin of the metastasis.

Clonality markers with a very high predictive power are immunoglobulin heavy (IGH) and K light chain (IGK) gene rearrangements in lymphoma. Occurrence of these rearrangements is not tumor-specific, all lymphocytes harbor unique immunoglobulin rearranged sequences. However, when a tumor originates from one of these lymphocytes, all lymphoma cells will harbor this unique rearranged sequence. Detection of these unique sequences can therefore help in discriminating multiple primary lymphomas from metastatic disease. In **chapter 3** IGH and IGK rearrangements were studied in patients with successive B-cell lymphomas to determine whether these lesions were primary tumors and recurrences or unrelated multiple primary lymphomas.

Most clonality assays focus on genomic DNA, however, human cells also contain multiple copies of mitochondrial DNA (mtDNA). Alterations in mtDNA have been described for multiple tumor types. In **chapter 4** the potential use of the mtDNA D310 marker for tumor clonality determinations is discussed.

In the last years targeted NGS has been introduced into multiple molecular pathology laboratories, enabling the analysis of many genes simultaneously. The accuracy and additional value of targeted NGS for determining the clonal relationship between two lung lesions of a patient is discussed in **chapter 5**.

In **chapter 6** the value of targeted NGS in the diagnostic workup of *BRCA1/2* gene mutation carriers with more than one tumor location was evaluated, using a custom made primer panel for the detection of mutations as well as copy number changes.

Part II - Molecular diagnostics in the context of hereditary testing

Familial Adenomatous Polyposis (FAP) is an inherited disorder characterized by the onset of multiple polyps throughout the colon, which can give rise to colon cancer. FAP is caused by germline *APC* mutations. Desmoid tumors (soft tissue tumors) can occur sporadically or can be a first manifestation of FAP, however, germline *APC* testing is not routinely performed in children with desmoid tumors. The vast majority of these tumors are caused by mutations in *APC* or *CTNNB1*, which are mutually exclusive. In **chapter 7** β-catenin immunohistochemistry and *CTNNB1* mutation analysis were used to identify possible *APC* germline mutation carriers among children with desmoid tumors.

Diagnostic strategies for selection of patients for Lynch syndrome (LS) counseling include MSI testing and/or immunohistochemical analysis of the MMR proteins in tumor tissue. **Chapter 8** discusses a pitfall in this screening strategy for a family with biallelic *PMS2* mutations.

Patients indicated as suspected of LS, based on the LS algorithm[17], are tested for germline mutations in the MMR gene(s). However, in 35% of these patients no germline mutations in the MMR genes are detected[20,21]. In **chapter 9** somatic MMR gene aberrations were studied in colorectal and endometrial cancers of suspected LS patients without germline MMR gene mutations.

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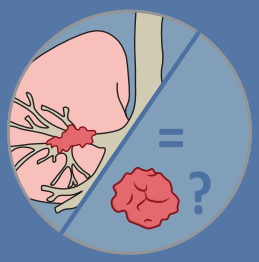

Chapter 2

CASE REPORT
Donor-transmitted metastasis of colorectal carcinoma in a transplanted liver

Zuzana Zelinkova, Willemina RR Geurts-Giele, Joanne Verheij, Herold Metselaar, Winand NM Dinjens, Hendrikus J Dubbink, Pavel Taimr

Transpl Int. 2012;25:e10-5.

Abstract

A 62-year-old man with alcoholic liver cirrhosis underwent liver transplantation. The transplantation went uneventful and the ultrasound imaging of the liver performed after transplantation did not show any abnormalities. Eighteen months later, an intra-hepatic focal lesion was found on ultrasound. A contrast-enhanced ultrasound revealed a lesion with a malignant pattern of contrast uptake. The histopathological and subsequent molecular pathological analysis concluded a colorectal metastasis of donor origin. The donor had no history of malignancy but no complete autopsy had been performed which illustrates the importance of the meticulous donors' screening. Transplanted patients carry a high risk of developing malignancy in general but donor related-tumors are very rare. The therapeutic considerations differ substantially between recipient- and donor-related malignancies. Therefore, considering the possibility of donor-related tumor by raising suspicion of malignant lesion with appropriate imaging and distinction from recipient-related malignancy by molecular analysis are crucial for proper therapeutic decision.

Introduction

Transmission of cancer from donor to recipient is a rare complication of solid organ transplantation. These donor-related tumors have been divided into two distinct entities, donor transmitted and donor derived tumors[1]. Donor transmitted tumors are defined as tumors present in the donor at the time of transplantation, in contrast to donor derived tumors that develop de novo in transplanted donor cells. To state the diagnosis of donor-related tumor, a good quality imaging and a molecular-pathological analysis are required. Here, we report a case of a donor transmitted metastasis of colorectal carcinoma in a liver transplant recipient in which the contrast-enhanced ultrasound (CEUS) directed further evaluation of a focal lesion detected in the transplanted liver.

Clinical history and imaging – part I

A 62-years-old man with alcoholic liver cirrhosis was placed on the waiting list for liver transplantation. During the period on the waiting list, he developed two intra-hepatic localizations of hepatocellular carcinoma (HCC) that were treated with radio-frequency ablation. Three years later, he underwent a liver transplantation with a deceased donor liver from a 69-years old female patient who died of cerebral vascular event and without a history of malignancy. The explanted liver of the patient showed three localizations of hepatocellular carcinoma. There were no macroscopic abnormalities noticed of the donor liver and the ultrasound performed after transplantation showed no lesions. The post-transplantation period went uneventful with a good graft function and the only long-term complication was the development of de novo diabetes mellitus.

Ultrasound at 18 months after transplantation showed lesion in the liver (Figure 1) of irregular shape, diameter of 5 cm and a homogenous hyperechogenic character. The overall aspect of the liver parenchyma and vasculature was normal. The differential diagnosis of this lesion was focal steatosis or recurrence of HCC. As a result of the patient's claustrophobia, a computed tomography (CT) scan instead of MRI was performed

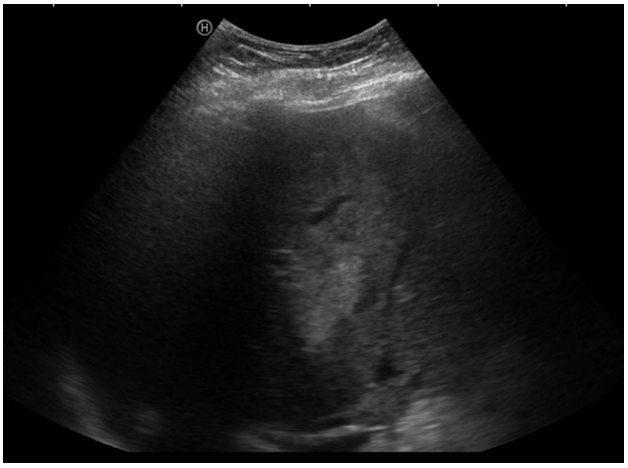

Figure 1. Ultrasound performed 18 months after liver transplantation showing an irregularly shaped hyperechogenic lesion of 5 cm in segment 8 in the transplanted liver.

and showed the lesion with no characteristic features of malignancy. CEUS using 2.5 ml Sonovue revealed an enhancement pattern suspicious of malignancy with a rapid arterial enhancement and a wash-out in the late venous phase within 2–3 min after administration of contrast (Figure 2). Therefore, an ultrasound-guided biopsy of the lesion was performed, showing a small fragment of tissue possibly of colonic origin. Therefore, a colonoscopy was performed, but no abnormalities were revealed. A CT scan repeated two months later showed a growth of the focal lesion from 5 to 7 cm and a new adjacent lesion of 2.7 cm. The histological evaluation of the repeated biopsy showed an adenocarcinoma compatible with a metastasis of colorectal carcinoma. The colonic origin was confirmed by additional immunohistochemical staining, cytokeratin 20 and caudal related homeobox-2 (CDX-2) were both positive in the tumor cells, and cytokeratin 7 was

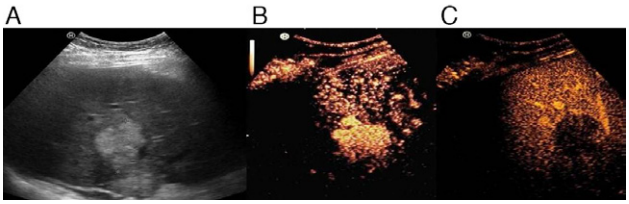

Figure 2. Contrast-enhanced ultrasound of the focal lesion in transplanted liver (a; B-mode) with a rapid arterial enhancement within few seconds after injection of 2.5 ml Sonovue contrast (b) and wash-out in the late venous phase at 2 min after contrast injection (c).

negative (Figure 3). The repeated colonoscopy being negative, the suspicion of donor-transmitted tumor was raised and molecular analysis was performed.

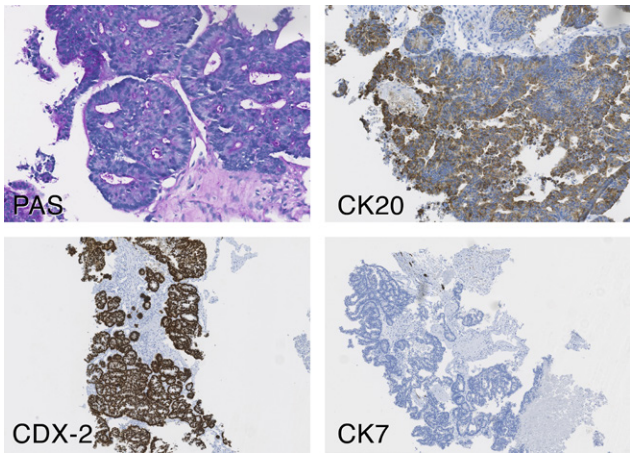

Figure 3. Biopsy of the focal liver lesion showing a mucus-producing adenocarcinoma (PAS staining), with positive staining for cytokeratin 20 and CDX-2 and no staining for cytokeratin 7, compatible with metastasis of a primary colon tumor.

Molecular analysis

First, tumor and normal tissues were genotyped. DNA was extracted from formalin-fixed paraffin-embedded (FFPE) tissues. A tissue area enriched for a high percentage of tumor cells and normal transplanted liver tissue were collected from sections by manual microdissection (Figure 4). Reference DNA was obtained from explanted FFPE liver tissue.

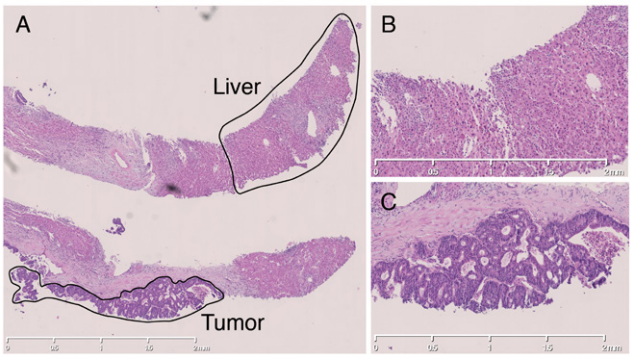

Figure 4. Tissue from the index patient (a), from which liver cells (b; transplanted liver) and tumor cells (c) were isolated.

Genotyping was performed by short tandem repeat (STR) profiling using the Powerplex 16 system (Promega). This system analyzes 15 STR loci and one sex chromosome marker. For each STR locus the number of repeats present was calculated using GeneMarker software (SoftGenetics). Results obtained with the green fluorescent labelled markers are shown in Figure 5. In Table 1, the repeat numbers are given for all samples examined.

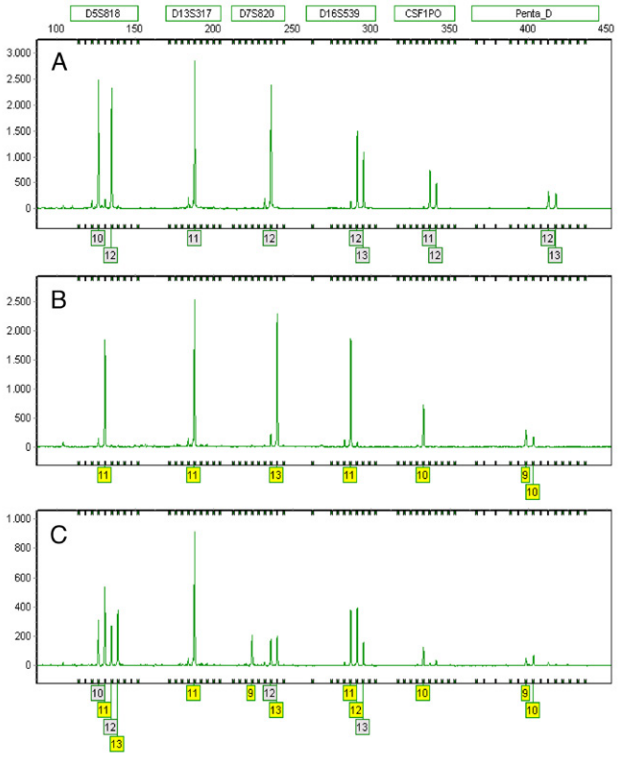

Figure 5. STR profiles of six markers (D5S818, D13S317, D7S820, D16S539, CSF1PO, Penta D) for explanted liver tissue (a), tumor tissue (b) and transplanted liver tissue (c). The transplanted liver sample (c) shows a combined donor (yellow boxes) and acceptor (grey boxes) STR pattern. All alleles of the tumor sample (b) are present in the transplanted liver sample and (except the alleles from marker D13S317) not in the explanted liver (a), indicating that the tumor cells are of donor origin. Several donor derived alleles in the transplanted liver are not present in the tumor tissue demonstrating DNA loss in the neoplastic cells (with markers D5S818, D7S820, and D16S539).

Comparison of the genotypes of tumor and explanted liver tissue showed that 13 markers display different number of repeats (Table 1). The major peaks of the transplanted liver tissue corresponded to the genotype of the tumor tissue and the minor peaks matched the genotype of the explanted tissue. These results strongly indicate that the tumor cells are

Table 1. Repeat numbers at short tandem repeat loci for explanted liver tissue, tumor tissue and transplanted liver tissue. The alleles of the tumor tissue are similar to the major peaks of the transplanted liver tissue, and different from the explanted liver tissue. This indicates that the tumor cells were derived from donor tissue.

Short tandem repeat locus		Amelogenin	D3S1358	TH01	D21S11	D18S51	Penta_E	D5S818	D13S317
Explanted liver		X, Y	15, 16	6,10	29, 30.2	12, 15	12, 17	10, 12	11
Tumor tissue		X	16, 18	6, 9.3	28, 30	15[a]	NA	11[a]	11
Transplanted liver	major peaks	X	16, 18	6, 9.3	28, 30	15, 16	12, 19	11, 13	11
	minor peaks	Y	15	-	29, 30.2	-	17	10, 12	-

Short tandem repeat locus		D7S820	D16S539	CSF1PO	Penta_D	vWA	D8S1179	TPOX	FGA
Explanted liver		12	12, 13	11, 12	12, 13	16, 17	8, 10	11	20, 21
Tumor tissue		13[a]	11[a]	10	9, 10	17, 18	12, 15	8	21, 23
Transplanted liver	major peaks	9, 13	11, 12	10	9, 10	16, 18	12, 15	8	21, 23
	minor peaks	12	13	-	-	17	8, 10	11	20, 21

[a]Loss of one allele in the tumor tissue

of donor origin. To further establish the female origin of the tumor cells, fluorescent in situ hybridization (FISH) of the X and Y chromosomes was carried out using Satellite Enumeration probes (DXZ1 and DYZ3, Poseidon), following standard protocols

All tumor cells as well as the transplanted liver cells showed either one or two X chromosomes, but no Y chromosome (Figure 6). The only cells harboring both an X and Y chromosome were infiltrating lymphocytes. These results underscore that the tumor cells are derived from female donor tissue.

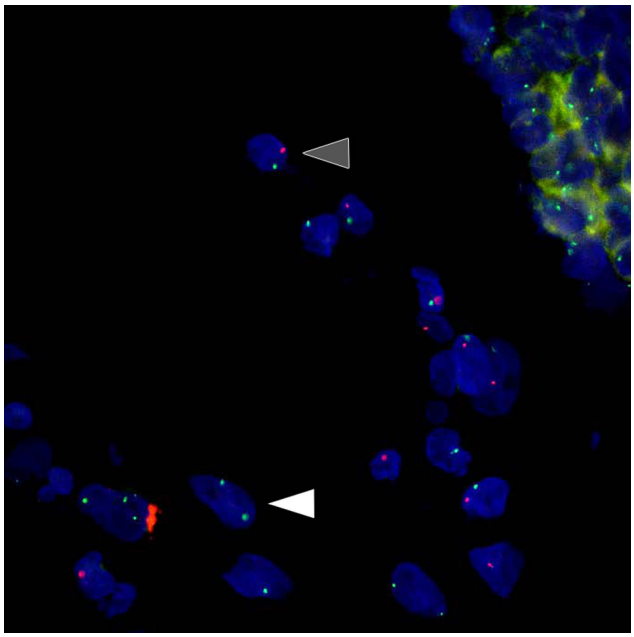

Figure 6. Fluorescent in situ hybridization was performed for the X and Y chromosomes, which are labeled green and red, respectively. A group of tumor cells is shown, together with some detached tumor cells (white arrowhead) and lymphocytes (grey arrowhead). The tumor cells have two X chromosomes, which confirms their female origin.

Clinical history and imaging – part II

Thus, 18 months after the transplantation, the patient was diagnosed with a donor-related metastasis of colorectal carcinoma in the transplanted liver. At further evaluation, no other localizations of this tumor were found. As a result of a recent myocardial infarction, cerebral stroke, and the development of psychiatric disorder with paranoid features, neither re-transplantation nor the local or systemic therapy could be offered. Patient died several months later, less than three years after the liver transplantation.

Discussion

We report a case of a donor-transmitted metastasis of colorectal carcinoma, identified 18 months after liver transplantation. This condition is very rare, the evaluation of the deceased donor-related tumor rate based on United Network for Organ Sharing (UNOS) registry (1994–2001) in almost 35 000 deceased donors being 0.04%[1]. Additional two cases of donor-transmitted tumors (glioblastoma and melanoma) were reported in the UNOS registry of the period between 2000 and 2005[2]. In the UNOS registry between 2005 and 2007, 15 tumors were confirmed in the solid organ transplantation and six recipients died as the result of a donor-transmitted disease[3]. The Israel Penn International Transplant Tumor Registry covering a period between 1965 and 2003 reports only two cases of donor-transmitted colon cancer[4].

Interestingly, not every diagnosed malignant tumor in the donor is necessarily transmitted to the recipient; the UNOS registry (2005–2007)[3] reporting one donor with proven colon cancer without transmission to the recipient. This case raises the question of the criteria for the donors' screening. The records of the donor showed no health problems but neither complete autopsy nor a CT scan have been performed as this is not part of the protocol. Two kidney recipients from the same donor have no signs of malignancy, which is not surprising given the specific metastatic pattern of colorectal carcinoma. Considering the still increasing age of donors and the high prevalence of colorectal carcinoma, the extent of the screening of the donors might need to be reconsidered with inclusion of a complete autopsy.

Other aspect of this report is the value of a new imaging modality, contrast-enhanced ultrasound. At CEUS, the liver metastases are characterized by a predominant arterial blood supply but hypovascular metastases can also be seen, especially in metastases of adenocarcinomas[5]. In this case, CEUS showed neoplastic features with rapid arterial enhancement and wash-out. However, the question in this case was the distinction of a secondary lesion from the recurrence of HCC, the latter being clinically the most likely diagnosis. This distinction was not possible with the CEUS image which is also the generally observed limitation of this technique[6]; however, a rapid wash-out of the contrast agent in a non-cirrhotic patient should raise the suspicion of a metastasis.

Finally, the diagnosis was revealed by histopathological examination. The morphology of the tumor corresponded to an adenocarcinoma, intestinal type which was confirmed by the additional staining. The clinical setting of negative colonoscopy prompted further molecular analysis. The techniques used were the STR profiling and chromosome FISH. The STR profiling has high sensitivity and is generally accepted for genotyping in forensic medicine[7].

Concerning the treatment and the prognosis of donor-derived tumors, the experience is limited. From the five donor-derived (four proven, one possible) cases reported in UNOS registry in 2007, three patients were re-transplanted with favorable clinical outcomes. Provided that the extra-hepatic localization of the tumor has been excluded, re-transplantation would be a curative treatment. As recipient-related metastatic malignancies or recurrence of HCC are much more common after liver transplantation and necessitate a different therapeutic approach, it is crucial to raise the suspicion of the donor-related malignancy and use molecular techniques to characterize the origin of the tumor.

Acknowledgements

We thank Hein Sleddens for performing the in situ hybridization.

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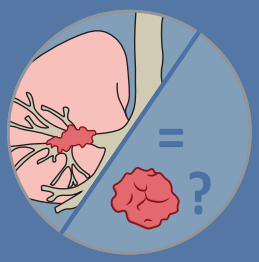

Chapter 3

Successive B-cell lymphomas mostly reflect recurrences rather than unrelated primary lymphomas

Willemina RR Geurts-Giele, Ingrid LM Wolvers-Tettero, Winand NM Dinjens, King H Lam, Anton W Langerak

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Abstract

Objectives: To address whether successive B-cell lymphomas, diagnosed within a 5- to 15-year interval, are recurrences or unrelated primary lymphomas.
Methods: Immunoglobulin heavy and K light chain gene rearrangements were studied using multiplex polymerase chain reaction fragment assays and sequence analysis in 61 patients.
Results: Clonal patterns of the multiple lymphomas from 36 patients were determined and classified accordingly: 30 recurrences, two possible recurrences, two different clones with a common origin, and two unrelated primary lymphomas.
Conclusions*:* Regardless of subtype, 89% to 94% of late B-cell lymphoma relapses were recurrences of the primary tumor. Therefore, routinely investigating the possible clonal relationship between successive lymphomas may not be warranted except for specific lymphoma subtypes such as diffuse large B-cell lymphomas.

Introduction

Malignant lymphomas are neoplasms of lymphocytes and their precursors. The main categories in the World Health Organization 2008 classification are Hodgkin lymphoma (HL) and non-Hodgkin lymphoma (NHL)[1]. After initial treatment, between 25% and 85% of patients with malignant lymphomas, depending on the subtype, show relapses following a period of complete remission (CR)[2-5]. The second and subsequent occurrence is usually considered a recurrence of the original tumor, even after many years of CR. However, a second occurrence of malignant lymphoma may also be an unrelated primary malignant lymphoma, especially after a long period of CR.

It is clinically relevant to differentiate between a recurrence and an unrelated primary lymphoma because the treatment strategy is usually different. Patients with recurrent lymphoma are usually treated aggressively, whereas patients with unrelated primary lymphomas generally receive the standard first-line therapies. Currently, little information is available with regard to the relative incidences of recurrent lymphoma and unrelated primary lymphoma. In previous studies, the percentage of patients with unrelated primary lymphomas rather than lymphoma recurrences ranged from 15% to 100%[6-12], as summarized in table 1. Limitations of these studies include their focus on particular lymphoma subtypes, the small sample sizes, or both. If the incidences of recurrences vs unrelated primary lymphomas were better documented, a better decision could be made on whether to perform molecular analysis to investigate the possible clonal relationship between successive lymphomas. To our knowledge, no such studies have been performed in a large clinical setting.

Using a single-center cohort consisting of all subtypes of B-cell lymphomas, we addressed whether successive B-cell lymphomas, diagnosed within a 5- to 15-year interval, are recurrences or unrelated primary lymphomas. Initially, 61 patients with multiple B-cell lymphomas were eligible for this study. Multiplex polymerase chain reaction (PCR) fragment assays were used to study rearrangements of the immunoglobulin heavy (IGH) and K light chain (IGK) genes in all lymphomas. Clonal patterns of the multiple lymphomas from 36 patients could be determined. When different fragment sizes were found in successive lymphomas of a patient, the rearrangements were sequenced to verify their different origin.

Table 1. Previous studies that examined successive b-cell lymphomas using molecular markers.

Study	Number of patients	Diagnosis primary lymphoma (number of patients)	Diagnosis relapse (number of patients)	Time interval (years)	(Suggested) recurrences	(Suggested) unrelated primary lymphomas	Percentage unrelated relapses
Ganzel et al[6]	6[a]	HL (3), CLL (2), low-grade B (1)	HL (3), DLBCL (2), MZL (1)	0-25	0	5	100
Obermann et al[7]	22	HL (21), DLBCL (1)	HL (21), DLBCL (1)	1-13	14	8	36
Lossos et al[8]	5	DLBCL (4), MCL (1)	cerebral DLBCL	3-12	0	5	100
De Jong et al[9]	13	DLBCL	DLBCL	4-17	11	2	15
Libra et al[10]	10	SLL (5), FL (3), MCL (2)	SLL (5), MCL (3), FL (2)	≥3	8	2	20
Nishiuchi et al[11]	5	DLBCL (4), FL (91)	DLBCL (3), FL (2)	>5	3	2	40
Mao et al[12]	26[b]	CLL	DLBCL	0-4	18	5	22

[a]For one patient, results were equivocal

[b]For three patients, results were not available

CLL: chronic lymphocytic leukemia; DLBCL: diffuse large B-cell lymphoma; FL: follicular lymphoma; HL: Hodgkin lymphoma; MCL: mantle cell lymphoma; MZL: marginal zone lymphoma; SLL: small lymphocytic lymphoma

Materials and Methods

Material selection

We studied a cohort of patients with a B-cell lymphoma, including HL, diagnosed between January 1, 1985, and September 21, 2011, at the Erasmus Medical Center, Rotterdam, the Netherlands. Patients were selected using the pathology laboratory information system Sympathy (Tieto, Helsinki, Finland). Using the Dutch pathologic anatomy national automated archive (PALGA), all PALGA codes containing *lymphoma* and excluding *bone marrow* or containing *Hodgkin* and excluding *lymphoma* were selected. Within these groups, a total of 163 patients had multiple lymphomas within a 5- to 15-year interval, with the most recent lymphoma being diagnosed in 2000 or later.

Subsequently, all patient histories were manually checked for the actual occurrence of multiple lymphomas and the availability of sufficient material for DNA analysis, resulting in the exclusion of 18 patients. Furthermore, patients with T-cell malignancies (n = 15) and patients treated outside the Erasmus Medical Center (n = 51) were excluded. The remaining 79 patients were categorized according to the diagnosis of the most recent lymphoma into one of the following groups: diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL), primary cutaneous follicle center lymphoma (PCFCL), marginal zone lymphoma (MZL), mantle cell lymphoma, chronic lymphocytic leukemia (CLL), HL, or NHL not otherwise specified (NHL-NOS). Remission statuses were derived from the hospital records. These statuses were determined according to the internationally accepted criteria, the most recent from Cheson et al.[13].

Of these patients, all available lymphoma tissues were included in the analysis, also when diagnosed outside the specified interval. All diagnoses of lymphoma were reviewed by an experienced hematopathologist (K.H.L.). If necessary, additional immunohistochemistry was performed and patients were recategorized accordingly. Patients were excluded when stainings (n = 8) or formalin-fixed, paraffin-embedded (FFPE) tissue blocks (n = 8) were not available. Furthermore, another two patients were excluded because they eventually did not fulfill all selection criteria. In the end, a total of 61 (37%) of the initially selected 163 patients were included for analysis, resulting in 183 tissue samples in FFPE blocks.

DNA isolation

From FFPE tissue blocks, 5 to 30 sections of 4 mm were cut depending on the size of the tissue. Tumor tissue was manually microdissected from 5 to 15 hematoxylin-stained sections when the tumor percentage was below 5% or when multiple lymphomas were present in one tissue block. Additional DNA was isolated from frozen tissue or dissected from routine cytological specimens (if present) when distinct rearrangements were found in successive lymphomas of a patient.

DNA was extracted using proteinase K (Roche Diagnostics, Indianapolis, IN) and 5% Chelex 100 resin (Bio-Rad, Hercules, CA), as previously described[14].

Clonality analysis

To check the quality of the samples, we performed a multiplex control PCR as previously described[15] with undiluted DNA and 1:10 and 1:50 dilutions. The dilution with the best results in agarose gel electrophoresis was selected for further analysis.

Multiplex BIOMED-2 PCR fragment assays were used to study rearrangements of the IGH and IGK genes in all samples. These assays were performed according to standardized protocols and primers[15]. IGH framework 1, 2, and 3 assays (IGH-A, IGH-B, and IGH-C) were used to detect Vh-Jh rearrangements, and IGH-D was used for the detection of incomplete Dh-Jh rearrangements. Vk-Jk rearrangements and functionally inactivating recombinations involving Kde (Vk-Kde or intron-Kde) were detected using IGK-A and IGK-B assays, respectively. The fragments were detected by GeneScan analysis on an ABI 3130XL genetic analyzer (Applied Biosystems, Foster City, CA), and data were analyzed with Peak Scanner Software version 2.1 (Applied Biosystems). Results were scored manually following recently described guidelines[16]. When different PCR product sizes were found within multiple lymphomas of a patient or when results were ambiguous, analyses were repeated.

Sequence analysis

When distinct rearrangements were found in multiple lymphomas of a patient, the fragments were sequenced to verify their different origin. The IGH or IGK gene rearrangements were amplified using unlabeled multiplex BIOMED-2 PCR assays[15]. Subsequently, heteroduplex analysis was performed to identify the presence of one or more clonal bands[17]. If a sample showed multiple homoduplexes or when a-specific bands were present, bands were cut from the polyacrylamide gel and eluted prior to sequencing. Single clonal PCR products were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA) according to the manual.

Sequencing was performed using the consensus Jh primer for IGH fragments, Jk1-4 and Jk5 primers for IGK-A fragments, and the Kde primer for IGK-B fragments. The BigDye Terminator version 3.1 Cycle Sequencing Kit was used for sequencing, after which products were purified using the BigDye Xterminator Purification Kit (both from Applied Biosystems), according to the manuals. The labeled fragments were detected on an ABI 3130XL genetic analyzer (Applied Biosystems). Data were analyzed with CLC DNA workbench version 5.7 (CLC Bio, Aarhus, Denmark). The involved Vh or Vk family member was identified using the IMGT/V-Quest alignment tool, version 3.2.25 (www. imgt.org/IMGT_vquest/vquest). Next, a Vh or Vk family primer was used to perform a monoplex PCR reaction from which the product was bidirectionally sequenced.

When DNA quality was not sufficient to identify the Vh or Vk family member using a multiplex PCR approach, PCR reactions were performed with all V family primers in monoplex reactions. If necessary, multiplex or monoplex PCR products were cloned into a pGEM-T easy vector (Promega, Madison, WI). Single-colony PCR was performed on the positive clones; the PCR products were directly sequenced.

Data analysis and classification of results

Multiple lymphomas with an interval of at least five years were classified as (a) recurrences, when two or more independent rearrangements showed the same fragment size; (b) possible recurrences, when only one interpretable marker showed identical sizes; (c) different clones with a common origin, when fragment size differences as well as similarities between the lymphomas were found; (d) unrelated primary lymphomas, when differences were found between the lymphomas in one or two markers, with at least two interpretable markers; or (e) non-evaluable, if they did not fit the other categories.

Results

A total of 61 patients with multiple lymphomas diagnosed within a 5- to 15-year interval were selected for analysis. Of these patients, all available lymphoma tissues were included in the analysis. Several of these additional lymphomas were diagnosed within the specified interval, shortening the remission time between two consecutive occurrences of lymphoma. Figure 1 shows a chronological overview of the multiple lymphomas for the 36 patients with evaluable results. For these patients, the interval between any two consecutive lymphomas ranged from 2 to 12 years. All CRs, partial remissions, and additional lymphomas for which no tissue was available, established within the intervals between the multiple lymphomas, are included as far as known based on the hospital records.

IGH and IGK clonality analysis points to a high frequency of lymphoma recurrence

Rearrangements of IGH and IGK genes were analyzed in DNA isolated from 61 patients. In the first 11 samples, all rearrangements involving the variable domains of IGH and IGK were tested using the BIOMED-2 IGH-A, IGH-B, IGHC, IGK-A, and IGK-B assays. Targets IGH-C and IGK-A gave the best results, with relative small product sizes of 100 to 300 base pairs (bp). This corresponded to the results of the control PCR, which showed that DNA quality was often compromised. Therefore, IGH-C and IGK-A were selected as the first targets to be tested in all other samples. However, targets IGH-C and IGK-A did not provide sufficient information to assess whether tissues were clonally related for 30 patients. In these patients, targets IGH-A and IGK were additionally analyzed, with product sizes between 210 and 390 bp. For 15 patients, markers IGH-B and IGH-D were also analyzed, with product sizes between 110 and

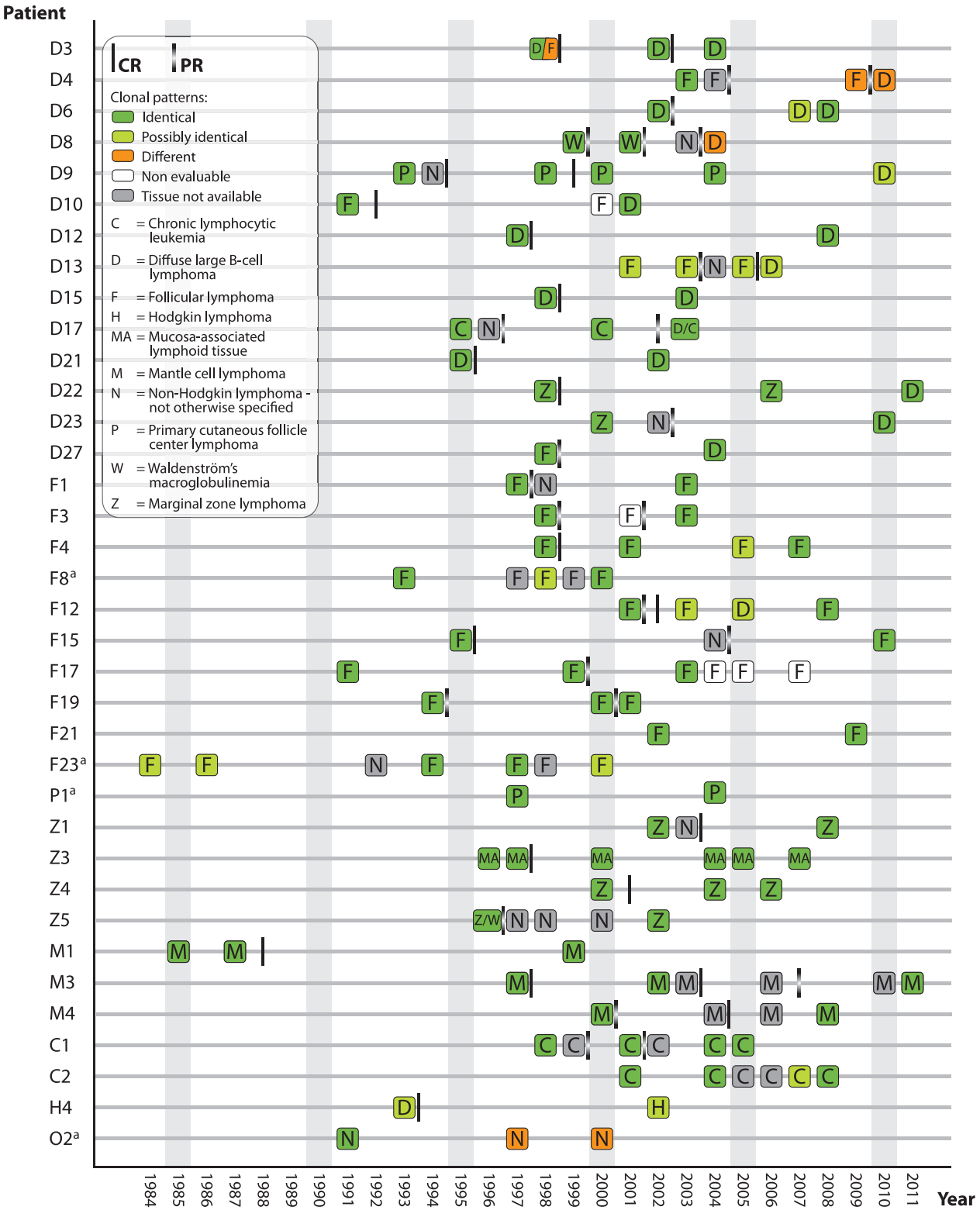

Figure 1. Chronological overview of the multiple lymphomas in the 36 patients with evaluable results. Complete remissions (CR) and partial remissions (PR) established within the intervals between the multiple analyzed lymphomas are indicated. Additional lymphomas diagnosed within these intervals for which no tissue was available are indicated in gray. Fragment analysis results are indicated by the different colors.

[a]No information about CR and PR available

420 bp. Some samples showed ambiguous results due to suboptimal DNA quality, whereas other samples showed polyclonal patterns probably caused by a very low percentage of lymphoma cells in the tissue sample. These patients were regarded as non-evaluable after analyzing two, four or six markers (in 13, 9, and 3 patients, respectively). An overview of the number of non-evaluable patients for the different lymphoma subtypes is provided in Table 2.

Table 2. Evaluable and non-evaluable patients for the different lymphoma subtypes.

	Total No.	Evaluable, No. (%)	Non evaluable, No. (%)
DLBCL	24	14 (58)	10 (42)
FL	16	10 (63)	6 (37)
PCFCL	4	1 (25)	3 (75)
MZL/MALT	4	4 (100)	0
MCL	3	3 (100)	0
CLL	3	2 (67)	1 (33)
HL	6	1 (17)	5 (83)
NHL-NOS	1	1 (100)	0

CLL: chronic lymphocytic leukemia; DLBCL: diffuse large B-cell lymphoma; FL: follicular lymphoma; HL: Hodgkin lymphoma; MALT: mucosa-associated lymphoid tissue; MCL: mantle cell lymphoma; MZL: marginal zone lymphoma; NHL-NOS: non-Hodgkin lymphoma not otherwise specified; PCFCL: primary cutaneous follicle center lymphoma

The samples of 18 patients showed IGH-C and IGK-A rearrangements of identical sizes (Figure 2a-2d). After also analyzing IGH-A and IGK-B and subsequently IGH-B and IGH-D, six and five patients, respectively, showed rearrangements of identical size in at least two evaluable markers. All of these patients showed two independent rearrangements of identical size except for patient F17, who showed identically sized fragments only with primer sets IGH-B and IGH-C, which target the same IGH rearrangement. For two patients, only one of the six markers was evaluable, showing rearrangements of identical sizes. Five patients showed one or more targets with rearrangements of different sizes in their samples (Figure 2e-2h). For these patients, all markers were analyzed. Figure 1 shows for each lymphoma whether the clonal patterns were scored as identical (at least two rearrangements of identical fragment size), possibly identical (only one rearrangement with the same fragment size), different, or non-evaluable. The results of IGH and IGK clonality analysis for all patients are provided in Table 3.

Sequencing of differently sized fragments shows evidence for clonally related and unrelated lymphomas

From the five patients with differently sized immunoglobulin- rearranged products in their

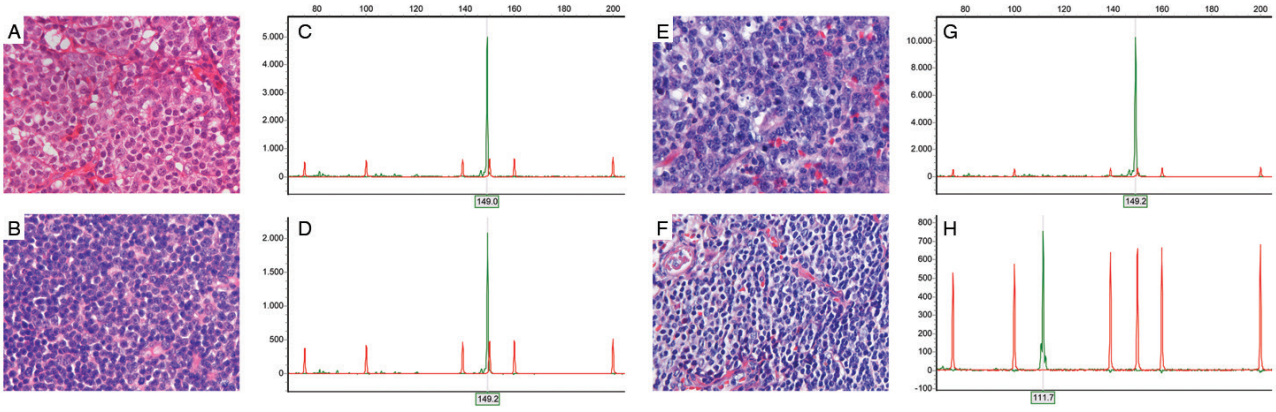

Figure 2. H&E stainings and clonality analysis results of IGH-C for the lymphomas of patients D17 and D8. Patient D17 presented with a DLBCL/aggressive CLL in 2003 (a) and a CLL in 1995 (b); both samples showed a fragment of 149 base pairs (c and d; DLBCL and CLL, respectively). Patient D8 presented with a DLBCL in 2004 (e) and WM in 1999 (f). These samples showed differently sized fragments of 149 (g; DLBCL) and 112 (h; WM) base pairs. H&E stainings are shown at 40 times.

CLL: chronic lymphocytic leukemia; DLBCL: diffuse large B-cell lymphoma; IGH: immunoglobulin heavy chain; WM: Waldenström macroglobulinemia

Table 3. Results of IGH and IGK clonality analysis for the multiple lymphomas of 61 patients [a].

Patient	Diagnosis lymphoma	Tissue	Year	IGH-A	IGH-B	IGH-C	IGH-D	IGK-A	IGK-B	Result
DLBCL										
D1	DLBCL	FFPE	2003			119		IP		Non evaluable
	DLBCL	FFPE	1992			[b]		[b]		
D2	DLBCL	FFPE	2010			[b]		[b]		Non evaluable
	DLBCL	FFPE	2005			IP		IP		
D3	DLBCL	FFPE	2004	319	253	118	210	144 (/151)	278	Differences found, additional analysis required
	DLBCL	FFPE	2002	[b]	(253)	118	[b]	144 (/149)	[b]	
	DLBCL	FFPE	1998	[b]	[b]	118	115/137	144 (/151)	[b]	
	FL	FFPE	1998	[b]	[b]	143	[b]	149 (/144)	[b]	
	DLBCL	Cytology	1998	319	253	118	141/245	144/151	278	
	DLBCL	Frozen	1998	319	253	118	[b]	144 (/151)	278	
D4	DLBCL	FFPE	2010	[b]	264	142	[b]	151	228/280 (231)	Differences found, additional analysis required
	DLBCL	Frozen	2010	330	264	IP (/116)	178	(217/223?)	228/280 (/231/277)	
	FL	FFPE	2009	331	265	IP	133/229	IP	228/280 (/231/277)	
	FL	Cytology	2009	331	265	MP	178	152	229/280 (/231/277)	
	FL	FFPE	2003	322	257	IP	[b]	IP	228/280 (/231)	
D5	DLBCL	FFPE	2010	[b]		IP		148	279	Non evaluable
	FL	FFPE	2010	(336)		IP		149	279	
	DLBCL	FFPE	2009	[b]		IP		[b]	279	
	FL	FFPE	2004	[b]		[b]		P	[b]	
D6	DLBCL	FFPE	2008			143		150		Recurrences
	DLBCL	FFPE	2008			144		[b]		
	DLBCL	FFPE	2007			[b]		150		
	DLBCL	FFPE	2002			144		151		
D7	DLBCL	FFPE	2006			IP		P	MP	Non evaluable
	DLBCL	FFPE	2000			136 (/153)		NR	284/370	
D8	DLBCL	FFPE	2004	[b]	(271)	149	240	149	209	Differences found, additional analysis required
	WM	FFPE	2001	344	247	112	[b]	150	[b]	
	WM	FFPE	1999	[b]	[b]	112	[b]	149	[b]	
	WM	Frozen	1999	312	247	111	257	150	402?	
D9	DLBCL	FFPE	2010			114 (/99)		[b]		Recurrences
	PCFCL	FFPE	2004			114 (/124)		152		
	PCFCL	FFPE	2000			114		152		
	PCFCL	FFPE	1998			114 (/153)		152		
	PCFCL	FFPE	1998			114		152		
	PCFCL	FFPE	1993			114		152		
D10	DLBCL	FFPE	2001	NE		P		141/191	238 (/276)	Recurrences
	FL	FFPE	2000	[b]		[b]		[b]	[b]	
	FL	FFPE	1991	[b]		[b]		141 (/190)	238 (/276)	
D11	DLBCL	FFPE	2011			IP		IP		Non evaluable
	DLBCL	FFPE	2003			146		[b]		

Table 3. Continued.

Patient	Diagnosis lymphoma	Tissue	Year	IGH-A	IGH-B	IGH-C	IGH-D	IGK-A	IGK-B	Result
D12	DLBCL	FFPE	2008	[b]		NE		152	283	Recurrences
	DLBCL	FFPE	1997	324		108		152	284	
D13	DLBCL	FFPE	2006	[b]	[b]	NR	NR	149	215 /282	Possible recurrences
	FL	FFPE	2005	[b]	[b]	87	[b]	150	[b]	
	FL	FFPE	2003	[b]	[b]	NE	[b]	149	[b]	
	FL	FFPE	2001	[b]	[b]	115/118	196	149	269	
D14	DLBCL	FFPE	2007	[b]	[b]	[b]	[b]	141	276	Non evaluable
	DLBCL	FFPE	1993	(373)	[b]	[b]	186/281	NR	[b]	
D15	DLBCL	FFPE	2003	NE	[b]	139		150	279	Recurrences
	DLBCL	FFPE	1998	[b]	[b]	139		150	[b]	
D16	DLBCL	FFPE	2004	[b]	[b]	IP	127	P	237/275	Non evaluable
	DLBCL	FFPE	1999	[b]	[b]	IP	NR	150	275	
D17	DLBCL or agressive CLL	FFPE	2003	336	271	149		145/195	272/276	Recurrences
	CLL	FFPE	2000	336	[b]	149		145/195	271/276	
	CLL	FFPE	1995	336	[b]	149		145/195	272	
D18	DLBCL	FFPE	2008	[b]	[b]	P	IP	P	P?	Non evaluable
	DLBCL	FFPE	2003	[b]	[b]	P?	138/143	P?	[b]	
	DLBCL	FFPE	2003	NE	[b]	P	159	P?	NE	
	DLBCL	FFPE	2000	NE	[b]	113	123/202	149/288	283	
D20	DLBCL & HL	FFPE	2008	[b]	[b]	(127/136)		149	[b]	Non evaluable
	DLBCL	FFPE	2003	[b]	[b]	IP		IP	[b]	
D21	DLBCL	FFPE	2002	[b]	(267)	IP	232	148	(217/277)	Recurrences
	DLBCL	FFPE	1995	[b]	[b]	[b]	232	148	[b]	
D22	DLBCL	FFPE	2011	343	277	143		149/198	[b]	Recurrences
	MZL	FFPE	2006	[b]	[b]	143		149/198	IP?	
	MZL	FFPE	1998	343	(277)	143		149/198	MP	
D23	DLBCL	FFPE	2010	332	265	123		262/281	[b]	Recurrences
	MZL	FFPE	2000			123		149/263/281	[b]	
D25	DLBCL	FFPE	2010	[b]	[b]	P		P	NR	Non evaluable
	FL	FFPE	2009	[b]	[b]	NR		143/147	227/278	
	FL	FFPE	2005	(313)	[b]	IP		P	NR	
D27	DLBCL	FFPE	2004	316	251	MP	[b]	149	[b]	Recurrences
	FL	FFPE	1998	[b]	251	115	[b]	149	(212)	
FL										
F1	FL	FFPE	2003			130		149/152		Recurrences
	FL	FFPE	1997			130		149		
F2	FL	FFPE	2011			119/P		288/P		Non evaluable
	HL	FFPE	2003			MP		NR		

Table 3. Continued.

Patient	Diagnosis lymphoma	Tissue	Year	IGH-A	IGH-B	IGH-C	IGH-D	IGK-A	IGK-B	Result
F3	FL	FFPE	2003	320		112		137/174/187	236/277/284	Recurrences
	FL	FFPE	2001	[b]		NE		NE	[b]	
	FL	FFPE	1998	[b]		112		137/174/187	236/277/284	
F4	FL	FFPE	2007	325		P		148	NR	Recurrences
	FL	FFPE	2005	IP (/325?)		P		149	NR	
	FL	FFPE	2001	325		P		150/P	NR	
	FL	FFPE	1998	326		IP		149	NR	
F5	FL	FFPE	2009			[b]		148		Non evaluable
	FL	FFPE	2002			P		P		
F7	FL	FFPE	2003			P		P		Non evaluable
	FL	FFPE	1994			IP		P		
F8	FL	FFPE	2000	[b]		130		144/149/194	281	Recurrences
	FL	FFPE	1998	[b]		(123)		144/149/194	NR	
	FL	FFPE	1993	IP		P		145/149	281	
F11	FL	FFPE	2002	[b]		NR		NR	[b]	Non evaluable
	FL	FFPE	2001	[b]		NR		[b]	[b]	
	FL	FFPE	1990	[b]		109		NR	[b]	
F12	FL	FFPE	2008			108		147/196		Recurrences
	DLBCL	FFPE	2005			IP		147/196		
	FL	FFPE	2003			IP		147/196		
	FL	FFPE	2001			108		147/196		
F15	FL	FFPE	2010	329	[b]	115	133	280 (/NR)	[b]	Differences found, additional analysis required
	FL	Frozen	2010	329	257	115	134	280	237	
	FL	FFPE	1995	[b]	[b]	NR	[b]	145/151	[b]	
	FL	Cytology	1995	329?	IP	115	MP	151 (/281)	237	
F16	FL	FFPE	2009			IP		IP		Non evaluable
	FL	FFPE	2004			IP		IP		
F17	FL	FFPE	2007	[b]	[b]	IP	245	P	[b]	Recurrences
	FL	FFPE	2005	318	NE	IP	MP	P	209/377	
	FL	FFPE	2004	MP	NE	IP	142	284/P	377	
	FL	FFPE	2003	[b]	262	120	[b]	P	[b]	
	FL	FFPE	1999	371	[b]	IP	165	284/IP (/149?)	230/377	
	FL	FFPE	1999	311/326	262	121	MP	IP (/149?)	IP?	
	FL	FFPE	1991	[b]	262	121	184	IP (/149?)	229	
F19	FL	FFPE	2001	312		IP		139	218/238/274	Recurrences
	FL	FFPE	2000	313		124 /IP		139	[b]	
	FL	FFPE	1994	313		IP		139	IP	
F20	FL	FFPE	2004			122/127		141/155		Non evaluable
	FL	FFPE	1998			[b]		104/148		
	FL	FFPE	1998			(133)		(149)		

Table 3. Continued.

Patient	Diagnosis lymphoma	Tissue	Year	IGH-A	IGH-B	IGH-C	IGH-D	IGK-A	IGK-B	Result
F21	FL	FFPE	2009	319		NE		121/148	282	Recurrences
	FL	FFPE	2002	319		P		121 (/149)	282	
F23	FL	FFPE	2000	[b]	[b]	NR	(202/233)	149	235	Recurrences
	FL	FFPE	1997	325	259	118	[b]	149	238	
	FL	FFPE	1997	[b]	(259)	NR	[b]	149	[b]	
	FL	FFPE	1994	325	259	117	[b]	NR	238	
	FL	FFPE	1986	[b]	[b]	117	[b]	[b]	[b]	
	FL	FFPE	1984	[b]	[b]	NR	[b]	[b]	238	
PCFCL										
P1	PCFCL	FFPE	2004	325	259	IP	152	148/200	237/241	Recurrences
	PCFCL	FFPE	1997	324	258	124	[b]	148/200	[b]	
	PCFCL	FFPE	1997	324	258	124	[b]	148/200	[b]	
P2	PCFCL	FFPE	2002	[b]		MP		NR	234	Non evaluable
	PCFCL	FFPE	1992	[b]		[b]		NR	(319)	
	PCFCL	FFPE	1992	325		146		148	238	
	PCFCL	FFPE	1989	[b]		MP		[b]	[b]	
P3	PCFCL	FFPE	2011	[b]		NR		NR	[b]	Non evaluable
	PCFCL	FFPE	2005	(308)		NR		NR	[b]	
P4	PCFCL	FFPE	2007	328		IP		NR	239/361	Non evaluable
	PCFCL	FFPE	1996	[b]		IP		NR	[b]	
MZL										
Z1	MZL	FFPE	2008			146/P		149/P (/198)		Recurrences
	MZL	FFPE	2002			146		149/198		
	MZL	FFPE	2002			146		149/198		
Z3	MALT	FFPE	2007			131		149/198		Recurrences
	MALT	FFPE	2005			131		149/198		
	MALT	FFPE	2005			131		148/198		
	MALT	FFPE	2004			131		149/197		
	MALT	FFPE	2004			131		149/198		
	MALT	FFPE	2000			131		149/197		
	MALT	FFPE	1997			131		149/198		
	MALT	FFPE	1996			131		149		
Z4	MZL	FFPE	2006			111		149/P		Recurrences
	MZL	FFPE	2004			111		149/P		
	MZL	FFPE	2000			111		149		
Z5	MZL	FFPE	2002			112		148		Recurrences
	MZL or WM	FFPE	1996			112		148		
MCL										
M1	MCL	FFPE	1999			130		153		Recurrences
	MCL	FFPE	1999			130		153		

Table 3. Continued.

Patient	Diagnosis lymphoma	Tissue	Year	IGH-A	IGH-B	IGH-C	IGH-D	IGK-A	IGK-B	Result
	MCL	FFPE	1987			130		153		
	MCL	FFPE	1985			130		153		
M3	MCL	FFPE	2011			[b]		NE		Recurrences
	MCL	FFPE	2011			146		149/198		
	MCL	FFPE	2002			146		149/198		
	MCL	FFPE	1997			146		149/198		
M4	MCL	FFPE	2008	335		133	[b]	150	[b]	Recurrences
	MCL	FFPE	2000	334		133	[b]	149	(279)	
CLL										
C1	CLL	FFPE	2005			118		151		Recurrences
	CLL	FFPE	2005			118		152		
	CLL	FFPE	2005			117		152		
	CLL	FFPE	2004			118		152		
	CLL	FFPE	2001			117		152		
	CLL	FFPE	1998			117		152		
C2	CLL	FFPE	2008			121/160		147/151		Recurrences
	CLL	FFPE	2007			121		[b]		
	CLL	FFPE	2004			121/159		147/150		
	CLL	FFPE	2001			121/160		147/150		
C4	CLL	FFPE	2003			[b]		[b]		Non evaluable
	MZL	FFPE	1994			105		150/155		
	MZL	FFPE	1993			105		150/155		
HL										
H1	HL	FFPE	2007	[b]		[b]		[b]	[b]	Non evaluable
	HL	FFPE	2006	[b]		[b]		NR	[b]	
	HL	FFPE	2005	[b]		IP		NR	[b]	
	HL	FFPE	2005	[b]		IP		NR	[b]	
	HL	FFPE	1997	[b]		IP		NR	[b]	
H3	HL	FFPE	2009			IP		IP		Non evaluable
	suspect HL	FFPE	1999			[b]		149		
H4	HL	FFPE	2002	[b]	[b]	[b]	[b]	197 (/NR)	237/278/285	Possible recurrences
	DLBCL	FFPE	1993	[b]	[b]	[b]	[b]	NR	[b]	
	DLBCL	FFPE	1993	[b]	279	142	250	197	[b]	
H5	HL	FFPE	2003	[b]		NR		NR	163	Non evaluable
	HL	FFPE	2000	[b]		MP		NR	[b]	
	HL	FFPE	1995	[b]		[b]		NR	[b]	
H7	HL	FFPE	2005			(147)		[b]		Non evaluable
	HL	FFPE	1999			(135)		[b]		
H10	HL	FFPE	2010			115		MP		Non evaluable
	HL	FFPE	2000			(123)		NE		

Table 3. Continued.

Patient	Diagnosis lymphoma	Tissue	Year	IGH-A	IGH-B	IGH-C	IGH-D	IGK-A	IGK-B	Result
NHL - NOS										
O2	NHL-NOS	Cytology	2000	[b]	[b]	109	[b]	[b]	[b]	Different clones with a common origin
	NHL-NOS	Cytology	2000	357	[b]	109/160/170	[b]	143/152/193/287	[b]	
	NHL-NOS	Cytology	2000	[b]	[b]	(109)	[b]	IP	[b]	
	NHL-NOS	FFPE	1997	[b]	[b]	109/160	IP	143/152/192	[b]	
	NHL-NOS	Cytology	1997	357	251/ 297/ 303	109/160/170	[b]	143/152/193 (/287)	264/284	
	NHL-NOS	FFPE	1991	MP	296/303	124/160/170	184	143/192/287	234	

[a]Fragment sizes mentioned in parentheses are (additional) minor peaks

[b]No fragment detected

CLL: chronic lymphocytic leukemia; DLBCL: diffuse large B-cell lymphoma; FFPE: formalin fixed paraffin embedded; FL: follicular lymphoma; HL: Hodgkin lymphoma; IGH: immunoglobulin heavy chain; IGK: immunoglobulin k light chain; IP: irregular polyclonal; MALT: mucosa-associated lymphoid tissue; MCL: mantle cell lymphoma; MP: multiple peaks; MZL: marginal zone lymphoma; NE: non-evaluable; NHL-NOS: non-Hodgkin lymphoma not otherwise specified; NR: not reproducible; P: polyclonal; PCFCL: primary cutaneous follicle center lymphoma; WM: Waldenström macroglobulinemia

multiple lymphoma samples, DNA was isolated from additional FFPE, frozen, and cytological tissue if available. All six IGH and IGK targets were analyzed in these extra samples. To check whether the differently sized fragments in the patients represented independent rearrangements, we also sequenced the fragments (Table 4).

Patient D3 showed an IGH-C fragment of 143 bp in the FL from 1998, whereas the DLBCL from 1998, 2002, and 2004 showed 118-bp fragments. After sequencing, the FL showed a dominant IGH-C fragment involving IGHV4-34 and a minor fragment involving IGHV3. This minor fragment was not visible during fragment analysis. All other samples from this patient showed only one fragment, identical to the IGHV3 rearrangement. The DNA from the FL was isolated from a tissue block containing a DLBCL/FL composite lymphoma. The minor fragment involving IGHV3 found in the FL is therefore most likely due to cells from the DLBCL. Because of the different rearrangements, this patient was classified as having unrelated primary lymphomas.

Patient D4 showed IGH-A and IGH-B fragments of 322 and 257 bp, respectively, in the FL from 2003 compared with 330 and 264 bp for the FL from 2009 and the DLBCL from 2010. All samples had an IGH-A or IGH-B fragment involving IGHV3. However, the sequence of the FL from 2003 was different from that of the other samples, including a different junction, resulting in the observed size difference. This patient showed two identically sized rearrangements involving the Kde element in all samples. One of these rearrangements involving VK1 was successfully sequenced in the FL from 2003 and the DLBCL from 2010 and turned out to be identical. Therefore, this patient was classified as having different lymphoma clones with a common origin.

Patient D8 showed an IGH-C fragment of 149 bp for the DLBCL from 2004, as well as 112-bp fragments for the Waldenström macroglobulinemia (WM) samples from 1999 and 2001. Sequencing revealed a fragment involving IGHV5- 51 for the DLBCL, and the WM samples showed a fragment involving IGHV3. All samples had an identically sized fragment of 150 bp with marker IGK-A. However, the DLBCL and WM rearrangements showed involvement of different V genes as well as different junctions. This patient was classified as having unrelated primary lymphomas.

Patient F15 showed differences for target

Table 4. Results of IGH and IGK clonality and sequence analysis for patients with differently sized fragments[a].

Patient	Diagnosis lymphoma	Year	Clonality analysis						Sequence analysis			
			IGH-A	IGH-B	IGH-C	IGH-D	IGK-A	IGK-B	IG	Complete rearrangement found?	Involved V gene	Junction
D3	DLBCL	2004/ 2002/ 1998	319	253	118	NR	144	278	IGH	yes, productive	IGHV3	CARVRGSFSLDYW
	FL	1998	[b]	[b]	143	[b]	149 (/144)	[b]	IGH	no	IGHV4-34	ND
D4	DLBCL/ FL	2010/ 2009	330	264	IP	178	151	228/ 280 (/231/ 277)	IGH	yes, productive	IGHV3	CATNTTIGPVSSSDHVSW
									IGK-B	yes, 2 rearrangements found	IGKV1-Kde (230 bp)	-21(10)-9[c]
											IGKV3-Kde (281 bp)	-17(12)-4[c]
	FL	2003	322	257	IP	[b]	IP	228/ 280 (/231)	IGH	yes, productive	IGHV3	CAKNVTSPADLDCW
									IGK-B	yes	IGKV1-Kde (230 bp)	-21(10)-9[c]
D8	DLBCL	2004	[b]	(271)	149	240	149	209	IGH	no	IGHV5-51	ND
									IGK-A	yes, productive	IGKV1-5	CQQYNSYPWTF
	WM	2001/ 1999	NR	247	112	257	150	NR	IGH	yes, productive	IGHV3	CARGGGAWDTS[d]
									IGK-A	yes, productive	IGKV1-17	CLQHDSYPRTF
F15	FL	2010	329	257	115	133	280 (/NR)	[b]	IGK-A	no	IGKV4-1	ND
	FL	1995	329?	IP	115	MP	151 (/281)	237	IGK-A	no	IGKV4-1	ND

[a]Fragment sizes mentioned in parentheses are (additional) minor peaks

[b]No fragment detected

[c]del 3' VK (ins) del 5' Kde

[d]Trp (W) not identified

Bp: base pair; DLBCL: diffuse large B-cell lymphoma; FL: follicular lymphoma; IG: immunoglobulin; IGH: immunoglobulin heavy chain; IGK: immunoglobulin k light chain; IP: irregular polyclonal; MP: multiple peaks; ND: not determined; NR: not reproducible; WM: Waldenström macroglobulinemia

IGK-A, with a 280-bp fragment for the FL from 2010 and a 151-bp fragment for the FL from 1995. Surprisingly, these samples showed an identically sized fragment on polyacrylamide gel and a nearly identical rearrangement involving Vk4-1. After reexamination of the fragment analysis results, a very low 281-bp peak was found for the FL from 1995. In addition, the samples showed identically sized IGH rearrangements. Therefore, these lymphomas were classified as clonally identical recurrences.

Patient O2 showed multiple fragments in targets IGH-C and IGK-A, some of which were mutually exclusive between the NHL from 1991 and the NHL from 1997 and 2000. This was true for the 124-bp and 109-bp fragments, respectively, for target IGH-C. For IGK-A, a 152-bp fragment was present in all samples except the NHL from 1991. However, in all samples, two and three additional identically sized fragments were found in targets IGH-C and IGK-A, respectively. Because of the complex rearrangement patterns, these samples were not sequenced. The patient was finally classified as having different lymphoma clones with a common origin.

No obvious relation exists between occurrence of unrelated lymphomas and time interval

Overall, clonal patterns of the successive lymphomas from 36 patients could be determined and were

classified accordingly: 30 recurrences, two possible recurrences, two different clones with a common origin, and two unrelated primary lymphomas. In both patients with unrelated primary lymphomas, the most recent lymphoma was diagnosed as a DLBCL. To study the possible association between occurrence of unrelated lymphoma and interval, we examined in more detail the intervals between the consecutive lymphomas. Of the 36 patients with evaluable results, two patients had an interval of more than 10 years between their consecutive lymphoma recurrences. Eighteen patients had an interval of 5 to 10 years between two consecutive occurrences of lymphoma: 15 recurrences, one possible recurrence, and two different clones with a common origin. All other patients had an interval of less than five years between their consecutive lymphomas.

Finally, we wanted to evaluate whether unrelated lymphomas occur in defined lymphoma subtypes. For two subtypes of lymphoma relapses, DLBCL and FL, we included at least 10 patients, with 14 and 10 patients with evaluable results, respectively. The DLBCL relapses were classified as follows: 10 recurrences, one possible recurrence, one different clone with a common origin, and two unrelated primary lymphomas. All FL relapses were recurrent lymphomas.

Discussion

Our study shows that, regardless of the subtype, 89% to 94% of late B-cell lymphoma relapses diagnosed within a 5-to 15-year interval are recurrences of the primary tumor. Only 2/36 (6%) successive lymphomas actually concerned clonally unrelated primary lymphomas. In both cases (patients D3 and D8), the most recent lymphoma was diagnosed as a DLBCL.

Although the most commonly used assay for determining whether two lymphomas are clonally related is fragment analysis of IGH and IGK rearrangements, there are some limitations to this approach. Ongoing somatic hypermutations are a common event in lymphomas, especially in FL. Only hypermutations resulting in deletions or insertions will be detected using fragment analysis. All FL in this study showed identical fragment sizes, suggesting that no deletions or insertions occurred in these lymphomas despite the large intervals. Differently sized fragments, however, can still represent the same rearranged fragment. In the current study, sequencing analysis was performed when differently sized fragments were found to see whether these differences truly reflect different tumor origins. The successive lymphomas of patient D4 showed differently sized IGH fragments both involving IGHV3 but with differences between the sequences and junctions. However, this patient showed two identically sized inactivating rearrangements involving the Kde element, one of which was confirmed by sequencing analysis. Despite the observed differences with the IGH marker, these lymphomas seem to have a common clonal origin. Furthermore, identically sized fragments found in two lymphomas can still represent two different rearrangements, as shown by Nishiuchi et al.[11]. The successive lymphomas of patient D8 showed an identically sized fragment with marker IGK-A. However, differently sized IGH fragments involving different V genes were found. The identically sized IGK fragment is due to the restricted IGK junctional heterogeneity, as different V genes and junctions were observed. Therefore, we suggest performing fragment analysis of at least two independent rearrangements when determining whether two occurrences of lymphoma are clonally related.

The two patients in this study with unrelated primary lymphomas showed different rearranged IGH fragments between their successive lymphomas. As only one IGH rearrangement was found for each lymphoma, the question arises whether these lymphomas are really different or could have a common origin possibly represented by a second undetected IGH rearrangement. Furthermore, clonally distinct relapses can also be recurrences from a subclone in the primary lymphoma. Previously, FL and DLBCL recurrences have been described for which the mutation patterns of the rearrangements were nearly identical to that of minor subclones in the primary FL[18,19]. In this study, however, both patients with unrelated primary lymphomas showed involvement of different IGHV genes in their successive lymphomas, making it unlikely that the relapses were subclones of the primary lymphomas.

For both patients with clonally unrelated primary lymphoma relapses, the morphology of the clonally distinct relapse was different from that of the primary lymphoma. Patient D3 presented with a DLBCL/FL composite lymphoma in 1998 and with DLBCL relapses after four and six years. The FL was clonally distinct from the multiple occurrences of DLBCL, which were all related. Patient D8 presented with WM in 1999 and 2001. In 2004, the patient was diagnosed with a relapse suggestive of transformation of the lymphoma to a DLBCL. However, this relapse was not clonally related to the WM. In 6/10 patients with DLBCL recurrences, the primary tumor was also of a different morphology. These patients showed clonally related DLBCL recurrences from FL, CLL, or MZL. Histologic transformations of multiple lymphoma subtypes to DLBCL have been reported extensively[20]. Thus, a DLBCL relapse from a morphologically different primary tumor by itself is not an indication of unrelated primary lymphomas.

Patient D17 presented with CLL in 1995 and 2000, as well as DLBCL/aggressive CLL in 2003; these lymphomas were clonally identical recurrences. In situ hybridization was performed to detect Epstein-Barr virus (EBV)–encoded RNA in the CLL and DLBCL samples of this patient, and a few positive nuclei were found. Because of the minimal amount of positive small nuclei, we are inclined to interpret this finding as a latent persistent EBV infection. However, we cannot exclude an oncogenic role in this respect, as other reports have demonstrated this[21,22].

Previous studies reported higher frequencies of unrelated primary lymphomas instead of recurrences; however, these studies focused on subtypes of lymphoma. The single-center cohort used in the current study resulted in 36 patients with clonal patterns that could be determined, including multiple subtypes of lymphoma. The largest subgroups were DLBCL and FL relapses, consisting of 14 and 10 patients, respectively. Within the subgroup of patients with DLBCL relapses, 2/14 patients had unrelated primary lymphomas. Correspondingly, de Jong et al.[9] studied DLBCL recurrences after 4 to 17 years; in 2/13 patients, they found evidence for clonally unrelated primary lymphomas. Nishiuchi et al.[11] studied three patients with DLBCL recurrences, one of whom was suggested to have unrelated primary lymphomas. All FL relapses turned out to be recurrent lymphomas. Unrelated primary lymphomas were not found in any subtype of lymphoma relapses besides DLBCL. This is in contrast to previous studies that report a considerable number of patients with unrelated primary lymphomas rather than recurrences. High incidences of unrelated primary lymphomas were suggested in cases of HL relapses[6,7] and central nervous system relapses[8]. For these subtypes of lymphoma, we unfortunately do not have enough data available. Overall, our results and those of others suggest that the incidence of unrelated primary lymphomas varies between different subtypes of relapses and primary lymphomas.

Even after a longer interval, we did not find a higher occurrence of unrelated primary lymphomas. Two patients had an interval of more than 10 years between their consecutive lymphomas; these patients were diagnosed with recurrences after a CR of 11 years. Furthermore, 18 patients who had an interval of 5 to 10 years between their consecutive lymphomas did not show unrelated primary lymphomas. The two patients with unrelated primary lymphomas had intervals of less than five years between their consecutive occurrences of lymphoma. It is also remarkable that one of these patients had only partial remission; apparently, this does not exclude the occurrence of a new unrelated primary lymphoma.

Twenty-five patients who had ambiguous results were classified as non-evaluable. DNA was isolated from FFPE tissue blocks due to the high availability of this tissue. Unfortunately, DNA quality was compromised as a result of fixation artifacts. The highest percentages of non-evaluable patients were in the PCFCL and HL subgroups—for 3 of 4 and 5 of 6 patients, respectively, clonal patterns of the successive lymphomas could not be determined. This could be explained by the combination of compromised DNA quality and the low amount of tumor cells in the tissues. The use of different patient tissues, especially for small-sized lymphomas, is therefore preferred.

In conclusion, late relapses of lymphomas are mostly recurrences of the primary tumor.

Therefore, routine investigation of the possible clonal relationship between two successive occurrences of lymphoma is not warranted given the high costs of the molecular assays. However, for specific subgroups of lymphoma, additional extensive molecular analysis might be valuable, especially for DLBCL recurrences.

To better document the incidence of unrelated primary lymphomas for specific lymphoma subtypes, a larger cohort is required, especially for lymphomas that are less common. Furthermore, it is important to perform sequencing analysis when differently sized fragments are found to confirm that these differences truly reflect independent rearrangements. To do this, it might be necessary to use patient tissues that are not formalin fixed to improve DNA quality.

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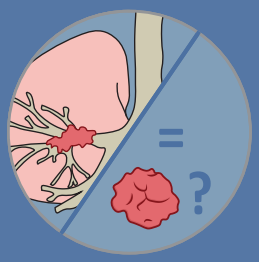

Chapter 4

Mitochondrial D310 mutation as clonal marker for solid tumors

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Abstract

Patients with multiple tumors, either synchronous or metachronous, can have metastatic disease or suffer from multiple independent primary tumors. While proper diagnosis of these patients is important for prognosis and treatment, this can be challenging using only clinical and histological criteria. The aim of the present study was to evaluate the value of mitochondrial D310 mutation analysis in diagnostic questions regarding tumor clonality for a wide range of tumor types. Sanger sequencing of D310 was performed on a diagnostic cohort of 382 patients with 857 tumors that were previously analyzed using routine molecular analysis on genomic DNA. The D310 mononucleotide repeat was frequently somatically mutated (56/321, 17%) in several tumor types, including breast, head and neck, gynecological, lung, colorectal, and skin tumors. For 84/327 (26%) patients, a D310 mutation was detected in at least one of their tumors; for these patients, D310 can be used to determine the clonal relationship between their multiple tumors. Clonality assessments based on mitochondrial DNA (mtDNA) and routine genomic DNA analysis were concordant in 52/73 (71%) patients. We conclude that D310 mutation status might aid in determining clonality of clinically challenging synchronous or metachronous tumors. To this end, next generation sequencing targeted genomic DNA assays should be complemented with mtDNA markers, such as the D310 repeat.

Introduction

When a patient presents with multiple tumors, either synchronous or metachronous, the question arises whether this is metastatic (recurrent) disease or, alternatively, the patient suffers from multiple primary tumors, as appears to be the case in 8% of cancer patients[1]. To distinguish between multiple independent primary tumors and metastatic disease is of prime importance for prognosis and treatment[2] but can be challenging, when only clinical and histological criteria are available. Since tumor cells differ from normal cells by the presence of clonal DNA aberrations, these can be used to determine whether or not a clonal relationship exists between multiple tumors within one patient[2-4].

Most molecular clonality assays focus on genomic DNA. Human cells, however, also contain numerous copies of mitochondrial DNA (mtDNA). Mutations in mtDNA initially result in heteroplasmic cells (cells with mutant and non-mutant mitochondrial DNA molecules). Upon cellular expansion, these heteroplasmic cells can achieve mutant DNA homoplasmy (all mtDNA molecules within one cell harbor the same mutation), as has been demonstrated in tumor models, human tumors, and tumor cell lines[5-8]. Apparently, homoplasmic mtDNA aberrations have been frequently found in human tumors[9], notably in a polymorphic cytosine mononucleotide repeat within the non-coding displacement loop (D-loop) region (D310)[10]. In several studies on different tumor types, mitochondrial DNA alterations have been used as a marker for clonality[11-14]. The aim of the present study was to evaluate for a wide range of tumor types whether or not D310 mutation analysis helps to solve diagnostic questions regarding tumor clonality.

For this study, we selected patients with multiple synchronous or metachronous tumors, for which the question of a clonal relationship was raised leading to routine molecular analysis on genomic DNA. We addressed the following questions: (1) Do these tumors have mtDNA D310 mutations? (2) Are the tumors clonally related based on mtDNA analysis and does this correspond to the clonality status assessed by routine genomic DNA analysis?

Materials and methods

We studied a cohort of patients with synchronous or metachronous tumors for which routine molecular clonality analysis on genomic DNA had been performed between January 2006 and April 2013 at the Erasmus Medical Center, Rotterdam, The Netherlands. All cases concerned patients for which pathologists or clinicians had previously submitted a request for molecular analysis in view of questions regarding diagnosis, prognosis, and/or patient treatment. For routine analysis, normal and tumor DNA had been extracted from formalin-fixed paraffin-embedded (FFPE) tissue blocks using proteinase K and, for extractions from 2009 onwards, 5% Chelex 100 resin, as previously described[15]. DNA was used in accordance with the Code of Proper Use established by the Dutch Federation of Medical Scientific Societies (https://www.federa.org/sites/default/files/digital_version_first_part_code_of_conduct_in_uk_2011_12092012.pdf). On these tumors, depending on the amount of tissue available and the tumor type, different combinations of routine molecular analyses had been performed, among which loss of heterozygosity (LOH) analysis, *TP53* mutation analysis following abnormal p53 immunohistochemical staining, and/or mutation analysis for other genes.

Of 466 patients eligible for inclusion in the study, 63 were excluded because no archival normal or tumor DNA was available, 17 because the original report was unavailable, and four because this was incomplete. In total, 857 tumors from 382 patients were included. Supplementary Table 1 shows an overview of all tumor details. Consecutive tumors in any single patient included have been numbered T1 to T7, in chronological order with T1 being the first diagnosed; in most cases this was the primary tumor.

PCR amplification of D310 was performed with normal and tumor DNA using Kapa 2G robust hotstart readymix (Kapa Biosystems, Woburn, MA) and M13-tailed custom-made primers (forward TGT AAA ACG ACG GCC AGT - TTG AAT GTC TGC ACA GCC AC and reverse CAG GAA ACA GCT ATG ACC - GGG GTT TGG CAG AGA TGT G). After purification using Exonuclease I and FastAP Thermosensitive Alkaline

Phosphatase (Fermentas, Thermo Fisher Scientific, Waltham, MA), PCR products were sequenced with M13 primers using the BigDye Terminator v3.1 kit (Applied Biosystems, Foster City, CA). Fragments were detected on a ABI 3730xl genetic analyzer (Applied Biosystems). D310 repeat length (nucleotide position 303–309) was evaluated by visual inspection using Mutation Surveyor v.3.24 software (SoftGenetics, State College, PA). An altered D310 repeat length in tumor DNA compared to patient-matched normal DNA was classified as a D310 mutation (either deletion or insertion). To exclude genomic DNA amplification, DNA isolated from mtDNA-less cells was used as a negative control (143B/206 ρ0, a kind gift of Dr. G.P. Comi, Dino Ferrari Centre, Neuroscience Section, Department of Pathophysiology and Transplantation (DEPT), University of Milan, Milan, Italy).

Results

Detailed results on the analysis of D310 in 857 synchronous or metachronous tumors of 382 patients are shown in Supplementary Table 1. Corresponding normal DNA could be evaluated in 332 patients and showed D310 repeat lengths of 6, 7, 8, or 9 cytosines (for 1, 187, 123, and 21 patients, respectively). Both normal DNA and DNA from the first tumor (T1) could be evaluated in 321 patients. A D310 mutation was found in 56/321 (17%) of T1, of which 11/85 (13%) in breast, 11/62 (18%) in head and neck, 4/35 (11%) in gynecological, 5/26 (19%) in lung, 8/25 (32%) in colorectal, and 3/19 (16%) in skin tumors (Figure 1). In 35/56 (63%) tumors, an insertion of one, two, or three nucleotides was found (in 25, 7, and 3 tumors, respectively); 21/56 (37%) tumors showed a deletion of one, two, or multiple nucleotides (18, 2, and 1 tumors, respectively). Of the 327 patients for whom D310 status could be determined, 243 (74%) showed tumors without D310 aberrations, while in 84 (26%) a D310 mutation was detected in one or more tumors (Table 1).

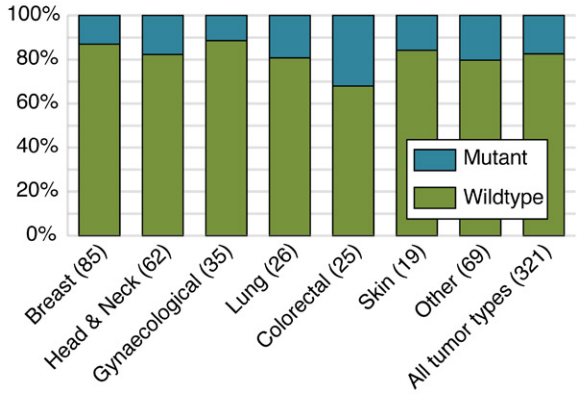

Figure 1. Percentage of D310 mutations in the chronologically first diagnosed tumors of all patients. The tumors are categorized by tumor type; after each tumor type, the number of tumors with an evaluable result is shown between parentheses.

Of the 84 patients with a D310 mutated tumor, 29 (35%) had clonally related tumors and 55 (65%) had multiple primary tumors based upon D310 mutation status. For 73 of these 84 patients, a final clonality status assessed by genomic DNA molecular clonality analysis was available, and in 52 (71%), mtDNA and genomic DNA results were concordant (Figures 2, 3 + Supplementary Table 2).

Discussion

We found that the D310 mononucleotide repeat in mtDNA is somatically mutated in 13% of breast tumors, 18% of head and neck tumors, 11% of gynecological tumors, 19% of lung tumors, 32% of colorectal tumors, and 16% of skin tumors. These results are in close agreement with previous studies in which D310 mutations were found in 11–28% of breast tumors, 0–16% of head and neck tumors, 3–26% of ovarian tumors, 0–13% of lung tumors, and 8–36% of colon tumors[10].

The identified D310 mutations were (nearly) homoplasmic, indicating that these mutations are present in the majority of the neoplastic cells and as a consequence must have occurred early during oncogenesis. Heteroplasmic D310 mutations have been reported in normal cells, achieving homoplasmy in tumor cells[6,12]. This suggests that D310 mutation status might provide an ideal marker for tumor clonality. We found in 84/327 (26%) patients with synchronous or metachronous tumors, for which the question of a clonal relationship was raised, a D310 mutation in at least one of the tumors. In such cases, D310 mutation status can be used to determine the possible clonal relationship between the tumors. In a large majority of patients (71%), clonality status assessed by mtDNA analysis and routine genomic

Table 1. Routine genomic versus mitochondrial DNA results for 84 patients with a D310 mutation in at least one of their tumors.

						Genomic DNA results				Mitochondrial DNA results						
Pt	**T1**	**T2**	**T3**	**T4**	**T5**	***TP53***	**LOH**	**Other**	**Conclusion**	**N**	**T1**	**T2**	**T3**	**T4**	**T5**	**Conclusion**
Concordant: 2 primary tumors																
9	Larynx, 2003	Pleura, 2006				Yes		Yes	T1≠T2	7	7	9				T1≠T2
22	Lung, 1994	Larynx, 2006					Yes		T1≠T2	8	7	8				T1≠T2
25	Breast, 2003	Breast, 2006				Yes	Yes		T1≠T2	8	9	8				T1≠T2
26	Adnex, 2006	Kidney, 2006					Yes		T1≠T2	NE	8	9				T1≠T2
36	Tonsil, 2006	Lung, 2007				Yes	Yes		T1≠T2	8	7	8				T1≠T2
51	Tonsil, 2007	Oesophagus, 2007	Lung, 2007			Yes	Yes		(T1=T2)≠T3	8	8	8	9			(T1=T2)≠T3
63	Cervix, 2006	Colon, 2007	Liver, 2007			Yes	Yes		T1≠T3 & T2≠T3	8	8	8	10			(T1=T2)≠T3
64	Lung, 2007	Adrenal gland, 2007					Yes		T1≠T2	8	10	8				T1≠T2
95	Lymphnode, 2000	Breast, 2008				Yes	Yes		T1≠T2	8	7	8				T1≠T2
118	Prostate, 2002	Skin, 2008					Yes		T1≠T2	7	7	8				T1≠T2
152	Breast, 1993	Breast, 2009					Yes	Yes	T1≠T2	8	7	8				T1≠T2
155	Breast, 1993	Breast, 2009					Yes	Yes	T1≠T2	9	9	8				T1≠T2
163	Tongue, 2008	Maxilla, 2009					Yes	Yes	T1≠T2	7	7	9				T1≠T2
185	Colon, 2006	Colon, 2010					Yes		T1≠T2	8	7	8				T1≠T2
200	Breast, 2010	Peritoneum, 2010					Yes		T1≠T2	8	8	7				T1≠T2
211	Rectum, 2002	Duodenum, 2010				Yes	Yes		T1≠T2	8	9	10				T1≠T2
213	Larynx, 2005	Oesophagus, 2010				Yes	Yes		T1≠T2	8	8	7				T1≠T2
216	Breast, 1998	Bladder, 2010					Yes	Yes	T1≠T2	8	8	9				T1≠T2
232	Breast, 2001	Breast, 2010				Yes	Yes		T1≠T2	8	8	7				T1≠T2
240	Mouth, 2010	Lung, 2010				Yes			T1≠T2	8	9	8				T1≠T2
250	Mouth, 2007	Lung, 2011				Yes	Yes		T1≠T2	9	9	8				T1≠T2
263	Abdomen, 1999	Pelvis, 2011					Yes		T1≠T2	8	7	8				T1≠T2
272	Mouth, 2009	Lung, 2011					Yes	Yes	T1≠T2	8	9	8				T1≠T2
314	Skin, 2011	Skin, 2011					Yes	Yes	T1≠T2	NE	9	8				T1≠T2
315	Vagina, 2011	Liver, 2011	Breast, 2011			Yes	Yes		T1≠(T2=T3)	8	8	9	9			T1≠(T2=T3)
352	Lung, 2012	Lung, 2012				Yes	Yes		T1≠T2	8	8	7				T1≠T2
367	Colon, 2012	Lymphnode, 2012				Yes			T1≠T2	9	10	7				T1≠T2

Table 1. Continued.

						Genomic DNA results				Mitochondrial DNA results						
Pt	**T1**	**T2**	**T3**	**T4**	**T5**	***TP53***	**LOH**	**Other**	**Conclusion**	**N**	**T1**	**T2**	**T3**	**T4**	**T5**	**Conclusion**
368	Lung, 2010	Pancreas, 2012					Yes	Yes	T1≠T2	8	7	8				T1≠T2
371	Breast, 2008	Ovary, 2011	Liver, 2012	Liver, 2012		Yes		Yes	T1≠(T2=T3=T4)	8	7	8	8	8		T1≠(T2=T3=T4)
373	Breast, 2010	Ovary, 2011				Yes			T1≠T2	8	9	8				T1≠T2
382	Mouth, 2012	Oesophagus, 2013				Yes			T1≠T2	7	10	6				T1≠T2
Concordant: clonally related tumors																
6	Nasopharynx, 2006	Maxillary sinus, 2006					Yes		T1=T2	8	9	9				T1=T2
41	Breast, 2002	Liver, 2007					Yes		T1=T2	9	7	7				T1=T2
59	Skin, 1996	Lung, 2007					Yes	Yes	T1=T2	8	9	9				T1=T2
62	Colon, 2004	Colon, 2006					Yes		T1=T2	9	8	8				T1=T2
93	Liver, 2006	Liver, 2008					Yes	Yes	T1=T2	8	7	7				T1=T2
137	Tonsil, 2008	Nasal cavity, 2009				Yes	Yes		T1=T2	8	7	7				T1=T2
143	Lymphnode, 2008	Epiglottis, 2008	Lung, 2009			Yes	Yes		T1=T2=T3	8	7	7	NE			T1=T2
177	Oesophagus, 2009	Oesophagus, 2009				Yes			T1=T2	7	8	8				T1=T2
184	Liver, 2007	Colon, 2010					Yes	Yes	T1=T2	8	10	10				T1=T2
196	Ovary, 2010	Endometrium, 2010						Yes	T1=T2	7	10	10				T1=T2
265	Larynx, 2010	Lung, 2011				Yes			T1=T2	8	10	10				T1=T2
270	Lymphnode, 2011	Lymphnode, 2011					Yes		T1=T2	8	9	9				T1=T2
299	Ovary, 2011	Uterus, 2011						Yes	T1=T2	8	7	7				T1=T2
326	Colon, 2006	Lung, 2012				Yes			T1=T2	8	9	9				T1=T2
344	Scrotum, 2012	Pleura, 2012					Yes		T1=T2	8	10	10				T1=T2
347	Skin, 2009	Lung, 2012					Yes		T1=T2	8	9	9				T1=T2
362	Breast, 2011	Lung, 2012	Skin, 2012			Yes			T1=T2=T3	8	10	10	10			T1=T2=T3
365	Breast, 2012	Colon, 2012						Yes	T1=T2	8	del	del				T1=T2
366	Colon, 2010	Lung, 2012				Yes			T1=T2	8	9	9				T1=T2
379	Colon, 2012	Bladder, 2013				Yes		Yes	T1=T2	8	7	7				T1=T2
380	Breast, 2011	Skin, 2013					Yes	Yes	T1=T2	7	8	8				T1=T2
Mitochondrial DNA: 2 primary tumors; genomic DNA: clonally related tumors																
3	Skin, 2003	Skin, 2003				Yes	Yes		T1=T2	7	7	8				T1≠T2

Table 1. Continued.

						Genomic DNA results				Mitochondrial DNA results						
Pt	**T1**	**T2**	**T3**	**T4**	**T5**	***TP53***	**LOH**	**Other**	**Conclusion**	**N**	**T1**	**T2**	**T3**	**T4**	**T5**	**Conclusion**
12	Breast, 2003	Peritoneum, 2004				Yes	Yes		T1=T2	NE	8	9				T1≠T2
104	Lung, 2008	Lung, 2008	Liver, 2008				Yes	Yes	T1=T2=T3	8	8	9	8			(T1=T3)≠T2
139	Lung, 2007	Lung, 2009					Yes		T1=T2	8	8	7				T1≠T2
237	Oesophagus, 2010	Oesophagus, 2010				Yes			T1=T2	8	9	8				T1≠T2
245	Colon, 2008	Lung, 2010				Yes		Yes	T1=T2	7	7	8				T1≠T2
268	Larynx, 2010	Lung, 2011				Yes	Yes		T1=T2	8	8	9				T1≠T2
334	Tonsil, 2008	Lymphnode, 2012					Yes		T1=T2	8	9	8				T1≠T2
350	Larynx, 2010	Larynx, 2010	Lung, 2012				Yes	Yes	T1=T2=T3	NE	7	8	7			(T1=T3)≠T2
Mitochondrial DNA: clonally related tumors; genomic DNA: 2 primary tumors																
27	Stomach, 2000	Pancreas, 2006				Yes	Yes		T1≠T2	8	7	7				T1=T2
219	Lung, 2007	Small intestine, 2010	Small intestine, 2010			Yes			T1≠(T2=T3)	9	10	10	10			T1=T2=T3
248	Breast, 2000	Breast, 2010					Yes		T1≠T2	9	8	8				T1=T2
262	Breast, 2003	Breast, 2011				Yes			T1≠T2	9	8	8				T1=T2
275	Pancreas, 2003	Liver, 2011				Yes			T1≠T2	8	9	9				T1=T2
324	Epiglottis, 2011	Lung, 2012				Yes			T1≠T2	8	9	9				T1=T2
346	Lymphnode, 2011	Palatum, 2012					Yes		T1≠T2	9	10	10				T1=T2
Discordant: complex																
178	Bladder, 2003	Lung, 2004	Small intestine, 2004				Yes	Yes	T1≠(T2=T3)	8	8	8	7			(T1=T2)≠T3
187	Oropharynx, 2004	Skin, 2006	Lung, 2010	Lung, 2010		Yes	Yes	Yes	T1≠T2≠T3≠T4	8	8	8	7	7		(T1=T2)≠(T3=T4)
306	Pharynx, 2011	Mouth, 2011	Larynx, 2011	Lung, 2011			Yes	Yes	T1≠T2≠T4	7	7	8	7	7		(T1=T3=T4)≠T2
316	Oesophagus, 1999	Oesophagus, 2002	Oesophagus, 2011			Yes		Yes	T2≠T3	NE	7	6	6			T1≠(T2=T3)
340	Lung, 1990	Bladder, 2007	Breast, 2009	Groin, 2012	Bladder, 2012	Yes			(T2=T4=T5)≠T3	8	10	8	8	8	9	T1≠(T2=T3=T4)≠T5
No comparison possible																
46	Breast, 1989	Breast, 2007							No conclusion	8	8	9				T1≠T2
69	Stomach, 2007	Colon, 2007							No conclusion	8	7	8				T1≠T2
91	Tonsil, 2006	Skin, 2008					Yes	Yes	T1=T2 (uncertain)	8	9	9				T1=T2
107	Colon, 1995	Colon, 1995	Vertebra, 2008						No conclusion	8	9	9	8			(T1=T2)≠T3
121	Thorax, 1996	Skin, 1998	Breast, 2008						No conclusion	8	8	8	10			(T1=T2)≠T3

Table 1. Continued.

						Genomic DNA results				Mitochondrial DNA results						
Pt	**T1**	**T2**	**T3**	**T4**	**T5**	***TP53***	**LOH**	**Other**	**Conclusion**	**N**	**T1**	**T2**	**T3**	**T4**	**T5**	**Conclusion**
126	Breast, 2000	Peritoneum, 2008							No conclusion	8	8	7				T1≠T2
173	Breast, 2009	Breast, 2009							No conclusion	9	7	9				T1≠T2
179	Skin, 2008	Breast, 2009					Yes	Yes	T1=T2 (uncertain)	8	9	8				T1≠T2
246	Oesophagus, 2011	Oesophagus, 2011							No conclusion	7	10	7				T1≠T2
259	Ovary, 2011	Endometrium, 2011	Ovary, 2011						No conclusion	8	10	9	10			(T1=T3)≠T2
372	Tongue, 2012	Pleural fluid, 2012						Yes	T1=T2 (uncertain)	8	9	8				T1≠T2

Patients are categorized according to the final results of both genomic and mitochondrial DNA analyses. Genomic DNA results are based on *TP53* mutation analysis, LOH analysis, and/or other analyses ("Yes" indicates that the particular analysis contributed to the final conclusion). Details about the genomic DNA analysis are provided in Supplementary Table 2. Mitochondrial DNA results show the D310 repeat length for all analyzed tumors.

Del: deletion; NE: non-evaluable; pt: patient; T1-T5: tumor 1-5

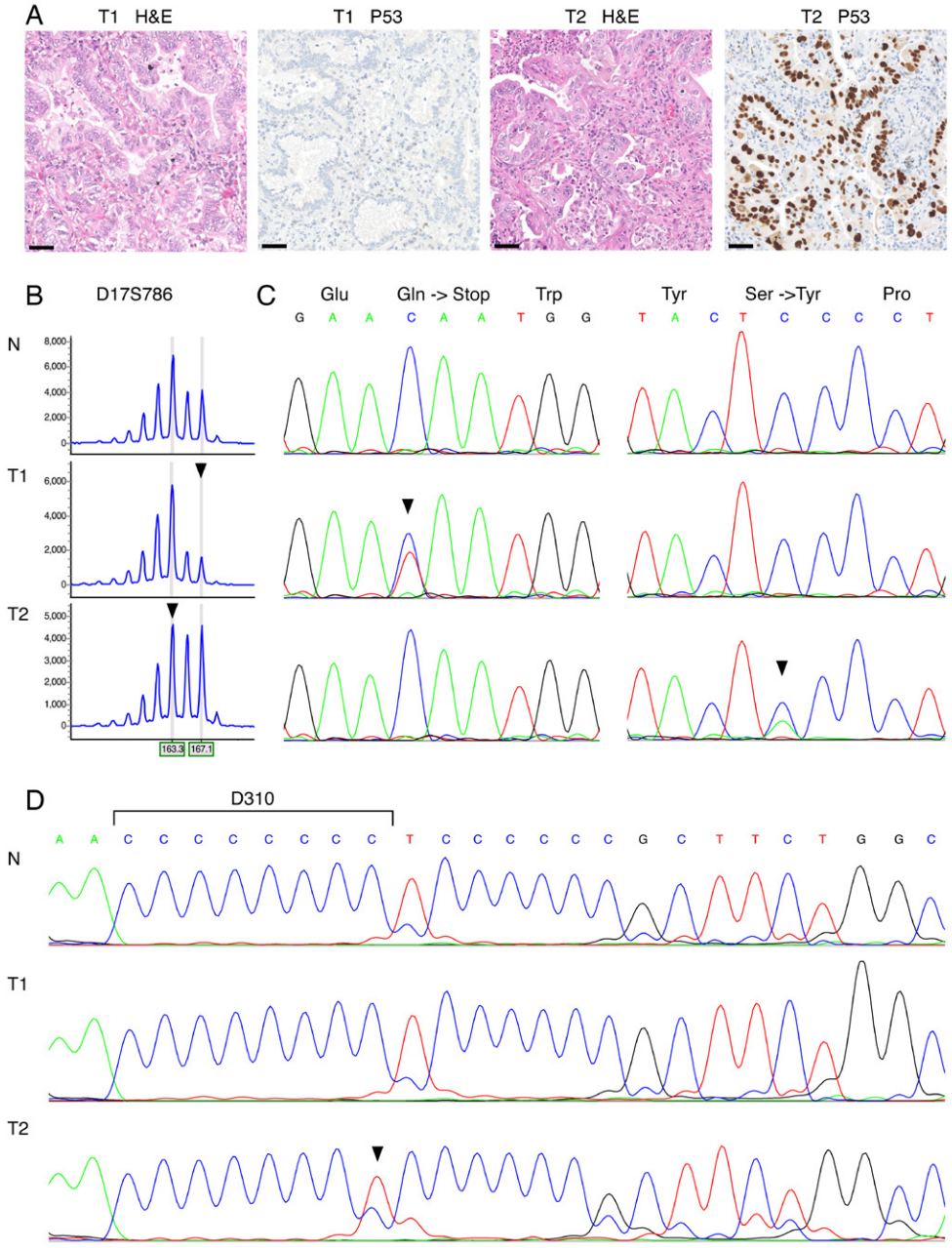

Figure 2. Routine genomic DNA and mitochondrial DNA results for patient 352, who was diagnosed with synchronous tumors of the right (T1) and the left lung (T2). (a) Both tumors were diagnosed as adenocarcinomas with a bronchioloalveolar growth pattern; T1 shows absence of p53 staining, whereas T2 shows clear nuclear p53 staining. Scale bars represent 50 µm. (b) Routine genomic DNA analysis was performed on DNA isolated from normal (N) and both tumor tissues (T1 and T2). LOH analysis of marker D17S786 (*TP53*) showed loss of the large allele in T1 and loss of the small allele in T2, indicated by arrowheads. The horizontal axis indicates the size of the DNA fragments in base pairs; the vertical axis indicates signal intensity. (c) Routine Sanger sequencing of *TP53* showed a p.Gln52* mutation only in T1, and a p.Ser127Tyr mutation only in T2, both indicated by arrowheads. (d) Sanger sequencing of mitochondrial DNA marker D310 showed an 8-cytosine repeat in normal DNA, no aberrations in T1, and a 1-bp deletion in T2, as indicated by the arrowhead. The results of routine genomic DNA and mitochondrial DNA analysis both indicate that T1 and T2 represent two primary tumors.

H&E: hematoxylin and eosin stain

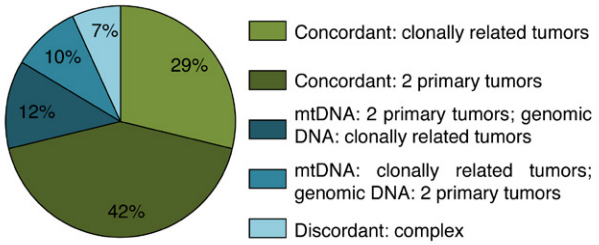

Figure 3. Clonality status assessed by mitochondrial DNA (mtDNA) results compared to routine genomic DNA results for 73 patients with a D310 mutation in one or more of their tumors.

DNA analysis were concordant.

Discordant results between clonality status assessed by mtDNA and genomic DNA analysis were found in 21/73 (29%) patients. Clonality assays on multiple tumors often result in some markers with concordant results but also markers with discordant results between the different tumors. Close scrutiny of individual markers is then necessary to decide whether the tumors are clonally related or not in view of the notion that genomic DNA analysis generates a likelihood that multiple tumors might be clonally related, but does not provide a definitive result. For 11 of our patients with discordant results, a highly likely diagnostic result was obtained because the tumors had a mutation in common, had mutually exclusive mutations, or the first tumor had a mutation that was not found in consecutive tumor(s). For these patients, the discordant mtDNA result was probably incorrect. Possible explanations are firstly that two primary tumors by chance may have acquired identical D310 mutations, secondly that de novo D310 mutations acquired during tumor progression result in clonally related tumors with different D310 mutations, and thirdly that intercellular or intracellular heterogeneity (heteroplasmy) in regard of D310 mutations is maintained during tumor development. For five patients, a likely diagnostic result was obtained because a mutation was only present in a consecutive tumor or the tumors showed common or different LOH status of five or more loci. For another five patients, the diagnostic result was weak, based on common or different LOH status of less than five loci. To reliably classify such tumors as clonally related or not, more informative genomic and/or mtDNA markers would be necessary.

Although D310 mutations are the most common mtDNA mutations in human cancer, other mtDNA deletions, insertions, and point mutations have been described[9]. Recently, next generation sequencing assays for mitochondrial DNA have become available[16]. The use of such assays for clonality analysis would result in the detection of more mutations and probably result in a higher predictive value. However, approximately 1.8 point mutations in somatic mtDNA have been found in only 60% of cancers[10], emphasizing the necessity to include analysis of genomic DNA as well. Mitochondrial DNA markers might be helpful when only a small number of cells are available, in view of the high number of mtDNA copies per cell compared to genomic DNA.

This study also has some limitations. Even though mtDNA is present in numerous copies per cell, facilitating amplification and analysis of a minute number of cells, no or an ambiguous D310 mutation analysis result was obtained for 55/382 (14%) patients. This was mostly due to an insufficient amount of DNA. For 11/84 (13%) patients with D310 mutations, a final clonality status assessed by genomic DNA analysis was not available, and for these patients, we were unable to compare mtDNA with genomic DNA results.

We conclude that D310 mutation status might aid in clonality determinations of clinically challenging synchronous or metachronous tumors, but as a single assay, has limited predictive value. To further evaluate the potential contribution of mtDNA markers to assessment of tumor clonality, we propose to include in existing next generation sequencing targeted genomic DNA assays mtDNA markers, such as the D310 repeat.

Supplementary material on the internet

Supplementary Table 1. Mitochondrial DNA results for all patients.
http://www.niekgeurts.nl/proefschrift/Chapter 4 -Supplementary Table 1.xls

Supplementary Table 2. Routine genomic versus mitochondrial DNA results for 84 patients with a D310 mutation in at least one of their tumors.
http://www.niekgeurts.nl/proefschrift/Chapter 4 -Supplementary Table 2.xls

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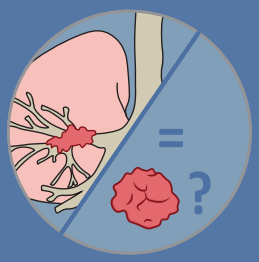

Chapter 5

CASE REPORT
Molecular diagnostics of a single multifocal non-small cell lung cancer case using targeted next generation sequencing

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Abstract

Histological and molecular subtyping of non-small cell lung cancer (NSCLC) is important for predicting survival and drug response in these patients. Up to 8% of NSCLC are multifocal and these tumor foci are often clonally related. Multiple foci can however also represent different primary tumors, with prognostic and therapeutic consequences. We describe a patient with multifocal NSCLC from which we obtained tissue from two separate lesions. With routine conventional molecular determinations, the clonal relationship between the two lesions was determined. In addition, targeted next generation sequencing with the Ion Torrent Personal Genome Machine (PGM) was performed to explore the accuracy and additional value of this relatively new technique. The two tumors of this patient showed different activating epidermal growth factor receptor (*EGFR*) mutations, *EGFR* amplification status, *TP53* mutation status, and loss of heterozygosity patterns. With the PGM, all conventional detected mutations were confirmed, and an additional variant of unknown significance in *ATM* was detected in one of the tumors. The multifocal NSCLC of this patient represents two unrelated primary tumors. Our results suggest that multifocal NSCLC should be considered as potentially multiple primary tumors. As the presence of activating *EGFR* mutations has important therapeutic consequences, *EGFR* testing should be performed on all tumor foci present. In the present case, targeted next generation sequencing using the PGM appeared to be accurate and comparable with conventional molecular determinations. However, the application of the PGM in routine pathology molecular diagnostics needs validation in larger series of cases.

Introduction

Lung cancer, with non-small cell lung cancer (NSCLC) as the most common type, is the leading cause of cancer mortality[1]. Histological and molecular subtyping of NSCLC is important for predicting survival and drug response. Patients with NSCLC harboring activating epidermal growth factor receptor (*EGFR*) mutations have a longer progression-free survival when treated with tyrosine kinase inhibitors (TKIs) compared to conventional chemotherapy[2]. Conversely, conventional chemotherapy is superior to TKI treatment in NSCLC patients without *EGFR* mutations.

Up to 8% of NSCLC are multifocal[3-6]. These tumors occur either synchronous (detected simultaneously) or metachronous (detected with a time interval). Multifocal lung tumors can be clonally related, but they can also represent multiple unrelated primary tumors. Previous studies showed that 7 to 64% of multifocal lung tumors are unrelated primary tumors[5-8]. This large range can be explained by inclusion of different subtypes of lung cancers and inclusion or exclusion of metachronous tumors. Furthermore, different combinations of molecular techniques were used to determine the clonal relationship between the tumors among which loss of heterozygosity (LOH) analysis and *EGFR*, *KRAS*, and *TP53* mutation determinations. Discriminating clonally related tumors from multiple primary tumors is of prime importance for appropriate treatment of the individual patient. It has been reported that patients with multifocal clonally distinct tumors may have a better outcome than patients with multifocal clonally related tumors[7]. Multifocality of a single primary NSCLC is probably an indication of the expanding or malignant potential of the lesion.

In the current study, we describe a patient with synchronous multifocal NSCLC. We addressed the question whether these foci are clonally related using routine molecular analysis. Furthermore, targeted next generation sequencing was performed to explore the accuracy and additional value of this relatively new technique in diagnostic questions regarding tumor clonality.

Clinical history

At the age of 69, the index patient was diagnosed with a multifocal lung tumor. The patient was a formerly healthy smoker (45 pack years) and presented with chest pain and weight loss. On a CT scan, we found three subpleural, nodular lesions; two in the left upper lobe and one in the right middle lobe of the lung. On a CT-guided cytologic biopsy, we did not succeed to get an adequate tumor sample so she was scheduled to have a video-assisted thoracoscopic procedure. A local excision of both tumors in the apex of the left lung was performed; a tumor of 15 mm in diameter was located at the ventral side (tumor 1) and a tumor of 17 mm in diameter was located at the dorsal side (tumor 2). At inspection of the pleural cavity, a diffuse nodular pattern was seen on both visceral and parietal pleura. Excision of the tumor in the right lung was not possible. Pathology revealed that both tumor foci of the left lung had comparable adenocarcinoma histology and both were TTF1 positive (Figure 1b), indicating their pulmonary origin. Patient was staged to have a pT4N0M1 NSCLC[9]. When molecular analyses showed two different *EGFR* exon 19 deletions in the two tumor foci, the patient was started on gefitinib (TKI). She showed a complete response which currently persists 32 months after start of the gefitinib treatment. CT scan 30 months after surgery showed no recurrence of disease.

Materials and methods

Normal and tumor tissues were manually microdissected from 5 to 15 hematoxylin-stained sections (4 µm) of formalin-fixed paraffin-embedded (FFPE) tissue blocks. DNA was extracted using proteinase K and 5% Chelex 100 resin, as previously described[10].

P53 and TTF-1 immunohistochemistry was performed with the mouse monoclonal antibodies Do-7 (Dako, Glostrup, Denmark) and SPT24 (Monosan, Uden, The Netherlands), respectively, according to standard protocols. To establish whether the *EGFR* locus was amplified in the tumor tissue, fluorescent in situ hybridization (FISH) was performed using *EGFR*/SE 7 probes (Kreatech, Amsterdam, The

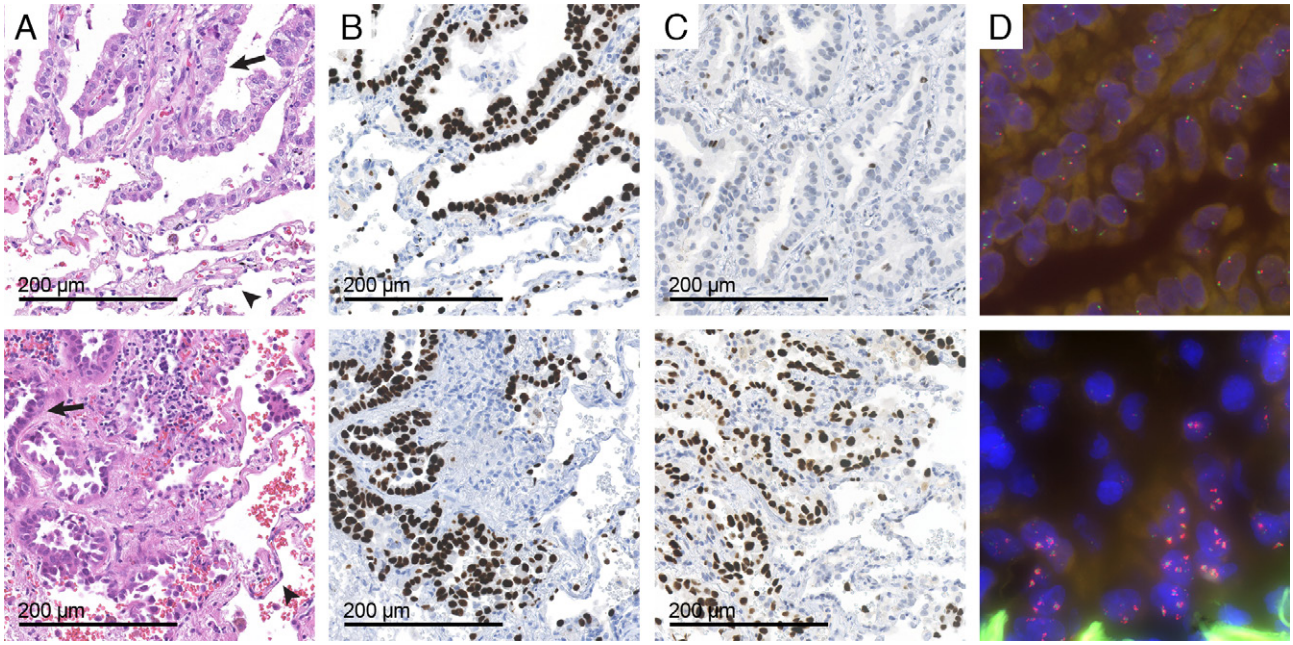

Figure 1. Hematoxylin and eosin (HE) stainings, TTF-1 and p53 immunohistochemistry and *EGFR* fluorescent in situ hybridization (FISH) results for tumor 1 (upper panels) and tumor 2 (lower panels). (a) HE stainings; the non-small cell lung cancer is indicated by arrows, normal lung tissue is indicated by arrowheads. Both tumor foci were diagnosed as adenocarcinomas. (b) TTF-1 staining was positive in both tumors, indicating their pulmonary origin. (c) p53 staining was absent in most cells of tumor 1; tumor 2 showed clear nuclear staining. (d) *EGFR* FISH shows the *EGFR* locus with red spots and the centromere of chromosome 7 with green spots. Two copies of *EGFR* were present in the cells of tumor 1, whereas tumor 2 showed amplification of the *EGFR* locus.

Netherlands), according to standard protocols.

Sequence analysis of *KRAS* codons 12, 13, 61, and 146, *EGFR* exons 18–21, and *TP53* exons 4–9 was performed with M13-tailed custom-made primers on tumor DNA. PCR amplification was performed with Kapa 2G robust hotstart readymix (Kapa Biosystems, Woburn, MA) according to the manual. PCR products were purified with Exonuclease I and FastAP Thermosensitive Alkaline Phosphatase (Fermentas, Thermo Fisher Scientific, Waltham, MA). The products were sequenced with M13 primers using the BigDye Terminator v3.1 kit (Applied Biosystems, Foster City, CA). Labeled fragments were detected on an ABI 3730xl genetic analyzer (Applied Biosystems). Data were analyzed with Mutation Surveyor v3.24 software (SoftGenetics, State College, PA). When variants were detected, normal DNA was sequenced to see whether the variants were tumor specific.

Snapshot analysis of *EGFR* was performed with the SNaPshot multiplex kit (Applied Biosystems) and custom made primers and probes to detect all *EGFR* hotspot mutations and deletions. The snapshot protocol was previously described[11].

LOH analysis was performed using normal and tumor DNA. FAM-labeled primers for the following microsatellite markers were used: D1S199, D3S1038, D3S1300, D5S421, D5S433, D8S133, D9S157, D9S1748, PTENCA, D10S541, D13S153, D13S263, D17S855, D17S1353, D17S786, D18S474, and D19S412. PCR amplification was performed as described for the mutation analysis. Labeled fragments were detected on an ABI 3730xl genetic analyzer (Applied Biosystems). Data were analyzed with Genemarker v1.85 software (SoftGenetics). Ion semiconductor sequencing on the Ion Torrent Personal Genome Machine (PGM) was performed with the Ion AmpliSeq Cancer Panel on normal and tumor DNA according to the manufacturer's protocols. In short, libraries were made using the Ion AmpliSeq Library Preparation Kit. Template was prepared using the Ion OneTouch Template Kit and sequencing was performed with the Ion Sequencing Kit v2.0 on an Ion 316 chip. Data were analyzed with Variant Caller v2.2.3-31149 (all Life Technologies,

Carlsbad, CA). Variants were called when the position was covered at least 500 times. Sequences of all primers and probes are available on request.

Results

Tumor 1 showed a heterogeneous p53 staining; most cells were negative but some scattered single cells showed slightly positive nuclear staining (Figure 1c). Tumor 2 showed positive nuclear p53 staining in almost all tumor cells, suggestive of a *TP53* mutation. *EGFR* FISH showed two copies of the *EGFR* locus in tumor 1 and *EGFR* amplification in tumor 2 (Figure 1d).

Table 1. Summary of the molecular data for both tumors of the index patient.

Gene/locus	Tumor 1	Tumor 2
Mutation analysis		
KRAS	WT	WT
EGFR	p.E746_A750del	p.L747_T751del
TP53	WT	p.S215I
LOH analysis[a]		
D1S199	ROH	ROH
D3S1038	LOH (short)	LOH (short)
D3S1300	LOH (short)	LOH (short)
D5S421	LOH (short)	ROH
D5S433	LOH (short)	ROH
D9S157	LOH (short)	LOH (short)
D9S1748	LOH (short)	ROH
D13S153	LOH (long)	LOH (long)
D13S263	LOH (short)	LOH (short)
D17S855	ROH	LOH (short)
D17S786	LOH (short)	LOH (long)
D18S474	LOH (long)	LOH (long)
D19S412	ROH	LOH (long)
FISH		
EGFR	Not amplified	Amplified
PGM - additional variant detected		
ATM	WT	p.Q3014X[b]

[a]Only informative markers are shown

[b]Detected in 94 out of 1,351 reads

FISH: fluorescent in situ hybridization; LOH: loss of heterozygosity; PGM: Ion Torrent Personal Genome Machine; ROH: retention of heterozygosity; WT: wild type

The molecular data for both tumor samples are summarized in Table 1. Sequencing analysis showed *EGFR* deletions in exon 19 in both tumor samples. Tumor 1 harbored a p.E746_A750del (c.2235_2249del15) and tumor 2 a p.L747_T751del (c.2240_2254del15) (Figure 2a). These deletions were confirmed by SNaPshot analysis (data not shown) and with the PGM (Figure 2b). Both tumor samples were wild type for *KRAS*. *TP53* exons 4 to 9 were sequenced in both tumor samples; tumor 1 was wild type and tumor 2 showed a p.S215I (c.644G>T) mutation in exon 6 (Figure 2c). This mutation was confirmed with the PGM (Figure 2d). Tumor 2 also showed LOH of marker D17S786, which is located near the *TP53* locus (Figure 3b). LOH analysis of ten chromosomes showed different LOH patterns between T1 and T2 at four chromosomes, including the *TP53* and *APC* loci (Figure 3). Furthermore, a variant of unknown significance (VUS) in *ATM* (p.Q3014X, c.9040C>T) was detected in a minority of the PGM reads of sample T2 (position covered 1,351 times of which 94 reads showed the variant). No other variants were detected with the PGM.

Discussion

The multifocal NSCLC of the index patient represents two unrelated primary tumors. The two tumors showed different activating *EGFR* mutations, which are early somatic mutations in NSCLC[12]. Several other differences were observed between the tumors, including *EGFR* amplification status, *TP53* mutation status, and LOH patterns, supporting that these tumors are different and unrelated entities.

In the present case, targeted next generation sequencing using the PGM is accurate; all mutations found by conventional molecular techniques were confirmed by the PGM. An additional VUS in *ATM* was detected in tumor 2 which, to our knowledge, has never been described before. This variant introduces a premature stop at codon 3014, leading to the truncation of 42 amino acids. The functional relevance of this variant is yet unknown. The variant was only found in a minor subset of the PGM reads, indicating molecular heterogeneity within this tumor.

PGM sequencing can be of additional value in tumor clonality determinations compared to routine

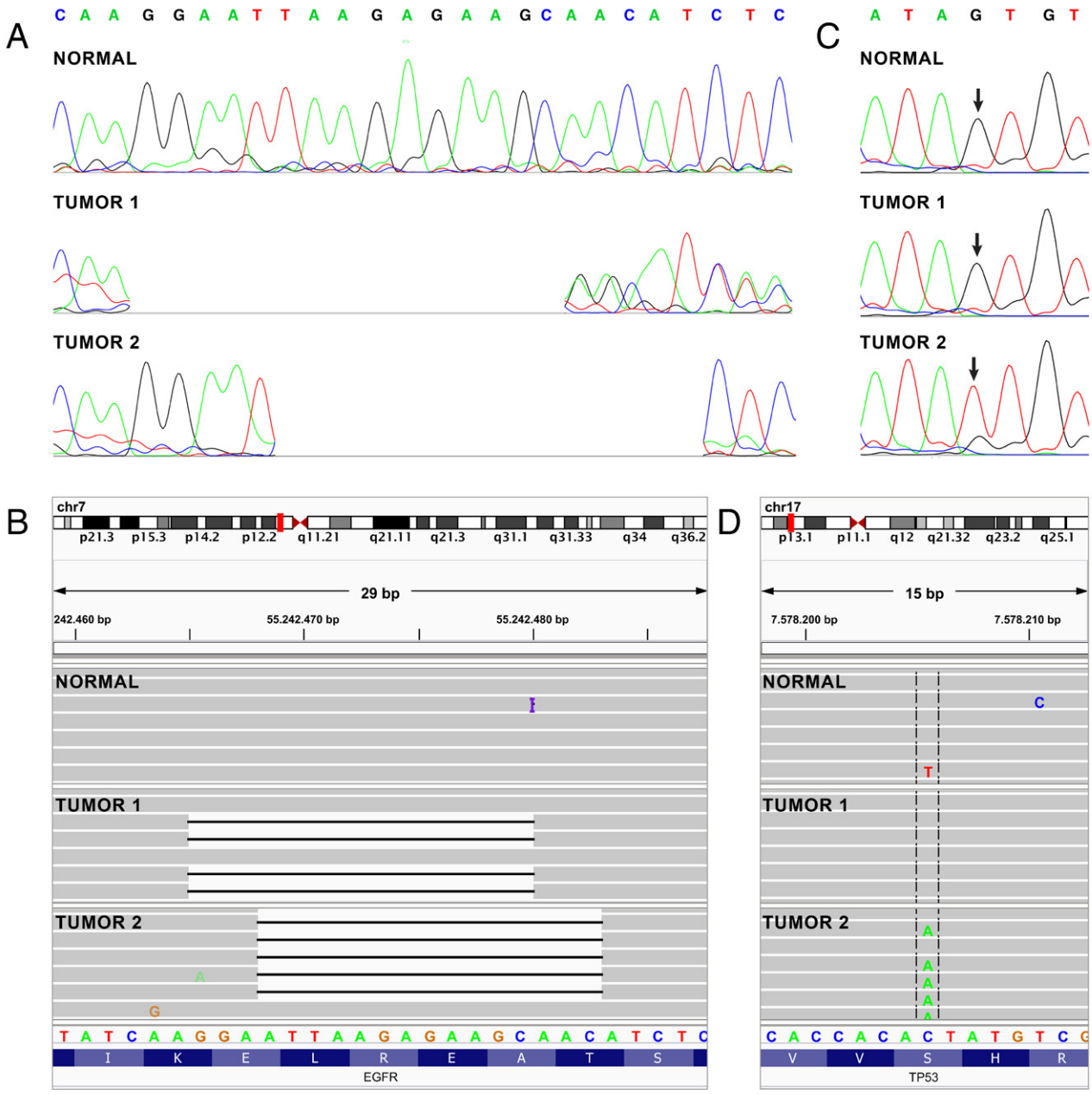

Figure 2. Sequencing analysis and Ion Torrent Personal Genome Machine (PGM) results for *EGFR* exon 19 and *TP53* exon 6. For the PGM data, each gray line represents an individual read; only aberrations from the wild type sequence are indicated. (a) Both tumors had an *EGFR* deletion, tumor 1 harbored a p.E746_A750del and tumor 2 a p.L747_T751del. (b) These deletions were confirmed with the PGM, the deletion is indicated by the black lines. (c) *TP53* sequencing revealed a p.S215I missense mutation only in tumor 2 (position is indicated by arrows). (d) This mutation was also confirmed with the PGM, shown in reverse complement.

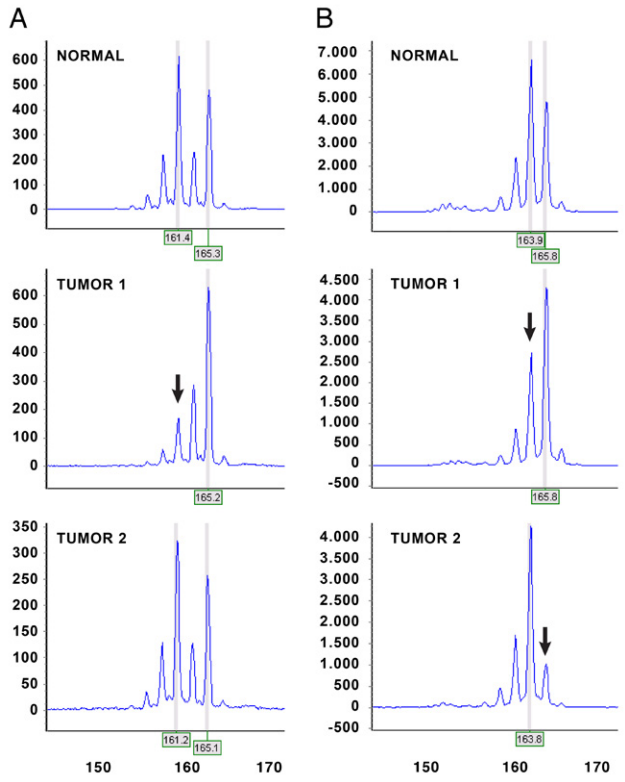

Figure 3. Loss of heterozygosity (LOH) results for the *APC* and *TP53* loci (markers D5S421 and D17S786, respectively). The alleles are indicated by gray lines, LOH is indicated by arrows. The horizontal axis indicates the size of the DNA fragments (in base pair); the vertical axis indicates signal intensity. (a) LOH of *APC* was detected in tumor 1; tumor 2 showed retention of both alleles. (b) LOH of *TP53* was detected in both tumors; tumor 1 showed loss of the short allele and tumor 2 showed loss of the long allele.

molecular analyses, especially when no mutations are detected using routine molecular techniques. Due to the low amount of DNA input necessary, the PGM can provide information about multiple genes even in cytology or small biopsy specimens. With only 10 ng of input DNA, the Ion Ampliseq Cancer Panel can detect 739 hotspot mutations in 46 oncogenes and tumor suppressor genes. Furthermore, PGM sequencing can successfully be performed with DNA retrieved from routine pathology FFPE tissue. Another advantage of the PGM is the high coverage, which enables the detection of variants down to 5% allele frequency. For conventional sequencing, a sample with 40 to 50% tumor cells is necessary for accurate detection of mutations, while for the PGM, 10% tumor cells in a background of normal, non-neoplastic cells, is sufficient. Additionally, a high coverage enables the detection of variants that are only present in a subpopulation of the tumor cells. This could be important in tumor clonality determinations, as a mutation present in one tumor might only be present in a subpopulation of the cells from the other tumor and would have escaped detection by conventional sequencing. However, the application of the PGM in routine pathology molecular diagnostics needs validation in a large cohort.

The index patient presented with two unrelated primary NSCLC, which raises the question whether the patient could be predisposed for developing this malignancy. The patient had a smoking history of 45 pack years. Warth et al.[7] reported that extensive smoking may increase the risk to develop multifocal, unrelated NSCLC. Although not significant, they found that patients with clonally unrelated NSCLC had higher numbers of pack years of smoking than patients with clonally related NSCLC. The genetic background can also predispose to the development of multiple lung tumors. It can be expected that next generation sequencing of constitutional DNA of NSCLC patients will aid in the identification of genetic risk factors.

Earlier studies reported that a high percentage of multifocal lung tumors are actually unrelated primary tumors[5,7,8]. In a recent study of Warth et al.[7], including 78 patients with synchronous, multifocal NSCLC, 28 patients (36%) are suggested to have separate primary tumors based on their unique molecular profiles. Our results and those of others indicate that multifocal NSCLC should be considered as potentially multiple primary tumors. Molecular testing is valuable to establish whether the tumors are clonally related. As the presence of activating *EGFR* mutations has important therapeutic consequences, it is indicated to perform *EGFR* testing on all tumor foci present.

Multifocal tumors consisting of multiple primary tumors possibly also have different *EGFR* mutation statuses. This poses a problem, as NSCLC harboring an activating *EGFR* mutation respond well to TKIs, whereas NSCLC without these mutations

respond better to conventional chemotherapy[2]. More research is necessary to establish whether NSCLC patients with a combination of *EGFR* mutated and *EGFR* wild type lung tumors will benefit from TKI treatment.

Acknowledgments

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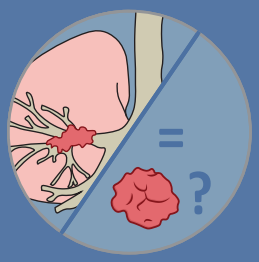

Chapter 6

Molecular determination of the clonal relationships between multiple tumors in *BRCA1/2*-associated breast and/or ovarian cancer patients is clinically relevant

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Abstract

Female *BRCA1/2* mutation carriers affected with breast and/or ovarian cancer may develop new tumor deposits over time. It is of utmost importance to know the clonal relationships between multiple tumor localizations, enabling differentiation between multiple primaries or metastatic disease with consequences for therapy and prognosis. We evaluated the value of targeted next generation sequencing (NGS) in the diagnostic workup of *BRCA1/2* mutation carriers with ≥2 tumor localizations and uncertain tumor origins. Forty-two female *BRCA1/2* mutation carriers with ≥2 tumor localizations were selected. Patients with inconclusive tumor origin after histopathological revision were 'cases'; patients with certain tumor origin of ≥3 tumors served as 'controls'. Tumors of cases and controls were analyzed by targeted NGS using a panel including *CDKN2A*, *PTEN* and *TP53*, hotspot mutation sites for 27 different genes and 143 single nucleotide polymorphisms for detection of loss of heterozygosity (LOH). Based on prevalence of identical or different mutations and/or LOH patterns, tumors were classified as 'multiple primaries' or 'one entity'. Conventional histopathology yielded a conclusive result in 38/42 (90%) of patients. Four cases and 10 controls were analyzed by NGS. In 44 tumor samples, 48 mutations were found; 39 (81%) concerned *TP53* mutations. In all four cases, the intra-patient clonal relationships between the tumor localizations could be unequivocally identified by molecular analysis. In all controls, molecular outcomes matched the conventional histopathological results. In most *BRCA1/2* mutation carriers with multiple tumors routine pathology work-up is sufficient to determine tumor origins and relatedness. In case of inconclusive conventional pathology results, molecular analyses using NGS can reliably determine clonal relationships between tumors, enabling optimal treatment of individual patients.

Introduction

Female *BRCA1/2* mutation carriers have a cumulative lifetime risk of developing breast cancer of 55-85% by the age of 70[1-4]. The cumulative lifetime risk of developing ovarian cancer varies between 15-60% for *BRCA1* and 10-35% for *BRCA2* mutation carriers[1-4]. Moreover, susceptibility for other cancers also seems to be increased in *BRCA1/2* mutation carriers[5,6].

It has been reported that *BRCA1*-associated breast cancers more frequently develop visceral metastasis and fewer bone metastases[7,8] and *BRCA2*-associated breast cancers tend to develop more lymph node metastases compared with sporadic breast cancer[8]. Metastatic sites of sporadic ovarian cancer mostly confine to the intraperitoneal cavity[9,10], whereas it has been described that *BRCA1/2*-associated ovarian cancer patients frequently (74%) present with visceral metastases to liver, lung and spleen[11]. Although this can be of some help, the non-specific metastatic patterns in *BRCA1/2*-associated breast and ovarian cancer patients impede careful differentiation between breast cancer, ovarian cancer and other tumor origins when multiple cancer localizations occur in one patient. It is of clinical importance, however, to make this distinction, as it guides surgical and chemotherapeutic treatment and determines prognosis[12,13].

A potentially helpful tool in determining clonal relationships between multiple tumors is DNA next generation sequencing (NGS)[14]. With NGS, selected genes known to be frequently mutated in specific tumor types can be analyzed. Additionally, single nucleotide polymorphisms (SNPs) can be analyzed to detect any DNA copy number changes present in the tumor cells. Identical molecular aberrations of different tumor localizations indicate a common tumor origin (e.g. metastatic disease), whereas different mutations and/or copy number changes in different tumor samples indicate two primary malignancies.

The aim of the current study was to evaluate the value of NGS in the diagnostic workup of *BRCA1/2*-associated breast and ovarian cancer patients with multiple tumor localizations.

Materials and methods

Patient selection: cases and controls

Patients at increased risk of breast and/or ovarian cancer visiting the Family Cancer Clinic of the Erasmus Medical Center Cancer Institute for counseling and surveillance programs are registered in an institutional ongoing database. All women provide written informed consent for registration of their clinical data and storage of genetic material (if relevant) for research purposes. From this database, we selected all female germline *BRCA1* or *BRCA2* mutation carriers with ≥2 synchronous or metachronous tumor localizations of which tumor material had been obtained by fine needle aspiration (FNA), biopsy or surgical excision. Tumor localizations of which no suitable material was available for histopathological or molecular analysis were excluded. Included were *BRCA1/2* mutation carriers with multiple tumors of which at least one was located in the breast or ovary. Inclusion and exclusion criteria are depicted in Table 1.

If possible, the origin of the tumor localizations was identified based on H&E staining. If tumor histology did not provide a conclusive diagnosis, immunohistochemical (IHC) staining was applied. Patients for whom the origin of one or more tumor localizations remained uncertain after histological and IHC evaluation were selected for NGS molecular analysis ('cases'). Patients with ≥3 tumor localizations of conclusive origin, based on histology and IHC, served as 'controls'. Controls were selected for NGS, as well, to validate the versatility of the NGS approach for tumor clonality determinations.

Conventional diagnostics

Tumor histology

Formalin-fixed paraffin-embedded (FFPE) tumor tissues were collected from the Department of Pathology of the Erasmus Medical Center Cancer Institute and from regional hospitals. Two pathologists specialized in breast and gynecological cancer (C.v.D., P.v.D.) independently reviewed haematoxylin and eosin (H&E) stained tissue sections of the tumor localizations for histology, with a subsequent consensus discussion.

Table 1. Inclusion and exclusion criteria.

Inclusion
• Women with a proven *BRCA1* and/or *BRCA2* mutation
• With ≥ 2 synchronous or metachronous tumor localizations[a]
• Tumor material available for next generation sequencing analysis
• (obtained by fine needle aspiration, histological biopsy or surgical excision)
• One of the 4 clinical scenarios:
1. Breast cancer and ovarian cancer
2. Breast cancer and second other tumor
3. Ovarian cancer and second other tumor
4. Breast cancer, ovarian cancer, and third or additional other tumor localizations
Exclusion criteria for 'other tumor localization'
• Hematological malignancies
• Dermatological malignancies (ie. melanomas, basal cell carcinomas)
• Ipsilateral lymph node metastases in the presence of breast cancer
• Premalignant lesions, such as ductal carcinoma in situ
• Contralateral breast cancer or second ipsilateral breast cancer, except in the presence of a third tumor localization
• Peritoneal tumor localization in the presence of ovarian cancer
– If reported that the ovarian cancer was growing per continuum into the peritoneal cavity
– Confining to the ipsilateral adnexa

[a]Isolated site of invasive cancer as diagnosed by radiological examination, intra-operatively or during pathological examination

Immunohistochemistry

IHC tissue markers were chosen according to the institutional protocol and depended on clinical and histological differential diagnosis of the origin of the various tumor localizations. Estrogen receptor (ER) was used as a breast cancer marker. IHC markers used for differentiation of ovarian cancer were cancer antigen 125 (CA125), Wilms' tumor 1 (WT1) and PAX-8, all known to be frequently expressed in ovarian cancer[15-17]. To differentiate with primary lung carcinoma, TTF-1 was used[18].

Molecular analysis

For cases and controls, p53 IHC was performed on all tumor tissues if FFPE tissue blocks were available. Nuclear expression of p53 in tumor cells was scored as either heterogeneous (no indication for *TP53* mutation), strong in all tumor cells (indication for missense *TP53* mutation) or absent in all tumor cells (indication for frameshift, nonsense, or splice site *TP53* mutation). For NGS analysis, normal and tumor tissues were manually microdissected from haematoxylin-stained tissue sections of FFPE tissue blocks or if unavailable, from original routine H&E, IHC stained sections or cytological preparations. DNA was extracted using proteinase K and 5% Chelex resin, as previously described[19]; DNA concentrations were measured with the Qubit 2.0 Fluorometer. To assess the quality of DNA amplification a multiplex control PCR was performed as previously described[20]; PCR products were analyzed on an agarose gel. All DNA samples were screened with the Ion Torrent Personal Genome Machine (PGM), with supplier's materials and protocols (Life Technologies, Carlsbad, CA, USA). A custom made primer panel was used, designed using Ion AmpliSeq Designer 2.2.1, for diagnostic use in clonality determinations of various tumor types including breast and ovarian cancer. Because this panel was designed for analysis of a broad range of tumor types, it includes genes frequently mutated in breast and ovarian cancer, as well as genes rarely mutated in these tumors. The panel targets almost the entire open reading frame of *CDKN2A*, *PTEN* and *TP53* (coverage 95-99%), multiple hotspot mutation sites for 27 different genes and 143 SNPs at 15 different loci for the detection of loss of heterozygosity (LOH, see Supplementary Table 1 for primer details). In total, the panel consisted of 254 amplicons with a mean amplicon size of 160 base pairs. With this panel, libraries were created using the Ion AmpliSeq 2.0 Library Kit. Template was prepared using the Ion OneTouch 2 with the Ion OneTouch 200 Template Kit v2 DL or using the IonChef with the Ion PGM Hi-Q Chef Kit. Sequencing was performed on an Ion 318v2 chip with the Ion PGM sequencing 200 kit v2 or the Ion PGM Hi-Q sequencing kit. Data was analyzed with Variant Caller v4.0 or v.4.4.2.1. Annotation of the variants was previously described[21]. For mutation detection, all exonic and splice variants with a variant percentage ≥20% were reported, excluding synonymous single-nucleotide variants and variants present in patient-matched normal tissue. Variants with a total coverage of <100

reads, reference coverage <10 reads, and/or a variant coverage of <5 reads for either the forward or reverse strand were excluded. For LOH analysis, SNPs with a total coverage of <100 reads or a strand bias (ratio forward:reverse reads not between 1:10 and 10:1 for reference and/or variant reads) were excluded. If a mutation was detected in one or more tumor samples of a patient, the specific locus was manually checked using the integrative genomics viewer (IGV) in normal DNA as well as all tumor samples of that patient. Furthermore, *TP53* was manually checked for mutations if no mutation was detected and IHC showed aberrant staining or was unavailable.

Samples for which the control PCR showed no signal for amplicons larger than 100 base pairs and for which NGS analysis showed <70% of reads on target and/or <70% of amplicons with at least 100 reads were defined low quality samples. For low quality samples with more than three variants, we focused on variants present in other tumors of the patient, or if not present, on *TP53* variants. For all low quality samples, mutations were confirmed by Sanger sequencing or by a second NGS run. For Sanger sequencing, primers from the AmpliSeq design were extended with M13 tails. PCR protocol was previously described[22], data was analyzed using Mutation Surveyor v.4.0 software (SoftGenetics).

Results

Patients

Fifty-six *BRCA1/2* mutation carriers with multiple tumor localizations were selected. Fourteen were excluded due to missing or unsuitable tumor material, leaving 42 women (39 *BRCA1*, 3 *BRCA2*) for analyses. Clinical classification of tumor origins was 'breast cancer + ovarian cancer' in 31 patients, 'breast cancer + other' in nine, 'ovarian cancer + other' in one, and 'breast cancer + ovarian cancer + other' in one woman (data not shown). Median number of tumor localizations was 2 (range 2-5), and median time from first to last cancer diagnosis was 5 years (range 0-23).

Conventional diagnostics

For 21/42 women (50%) the origin of the tumor localizations was conclusive based on histology only. In an additional 17 (40%) a conclusive diagnosis was reached after IHC for relevant markers. Ten of 38 women with conclusive outcomes based on histology and/or IHC had ≥3 tumor localizations (controls; eight *BRCA1* and two *BRCA2* mutation carriers).

In four women (10%) one or more tumor localizations remained of uncertain origin after histological and IHC evaluation (cases; all *BRCA1* mutation carriers).

Case no. 1 presented with tumors in the right and the left breast, and a tumor in the lung seven years later. Both breast tumors were diagnosed IDC of the breast based on HE staining. The lung tumor was diagnosed non-small cell carcinoma, however, conclusive diagnosis regarding the origin of the tumor was not possible based on HE and IHC (see Figure 1a for details).

Case no. 2 presented with a tumor in the ovary and a tumor in the breast six years later. The tumor of the ovary was diagnosed serous carcinoma of the ovary based on HE staining. The breast tumor was diagnosed adenocarcinoma based on cytological preparations; however, no tissue was available for IHC. Therefore, tumor origin could not be determined.

Case no. 3 presented with a tumor in the breast and peritonitis carcinomatosa 10 years later. The tumor in the breast was diagnosed IDC of the breast based on HE staining. The tumor cells found in the ascites were diagnosed adenocarcinoma based on cytological preparations. CA-125 and WT-1 IHC performed on de-stained cytological preparations was not conclusive, therefore, determining the site of the origin of this tumor was not possible.

Case no. 4 presented with a tumor in the breast and tumors in the retroperitoneal lymph nodes as well as in the ovary and uterus three years later. The tumor in the breast was diagnosed IDC of the breast and the tumor in the ovary and uterus serous carcinoma of the ovary, both based on HE staining. The tumor in the retroperitoneal lymph nodes was classified as a large cell carcinoma based on the HE staining. However, only a small biopsy was available, from which no tissue was left in the FFPE tissue block for additional analyses.

A

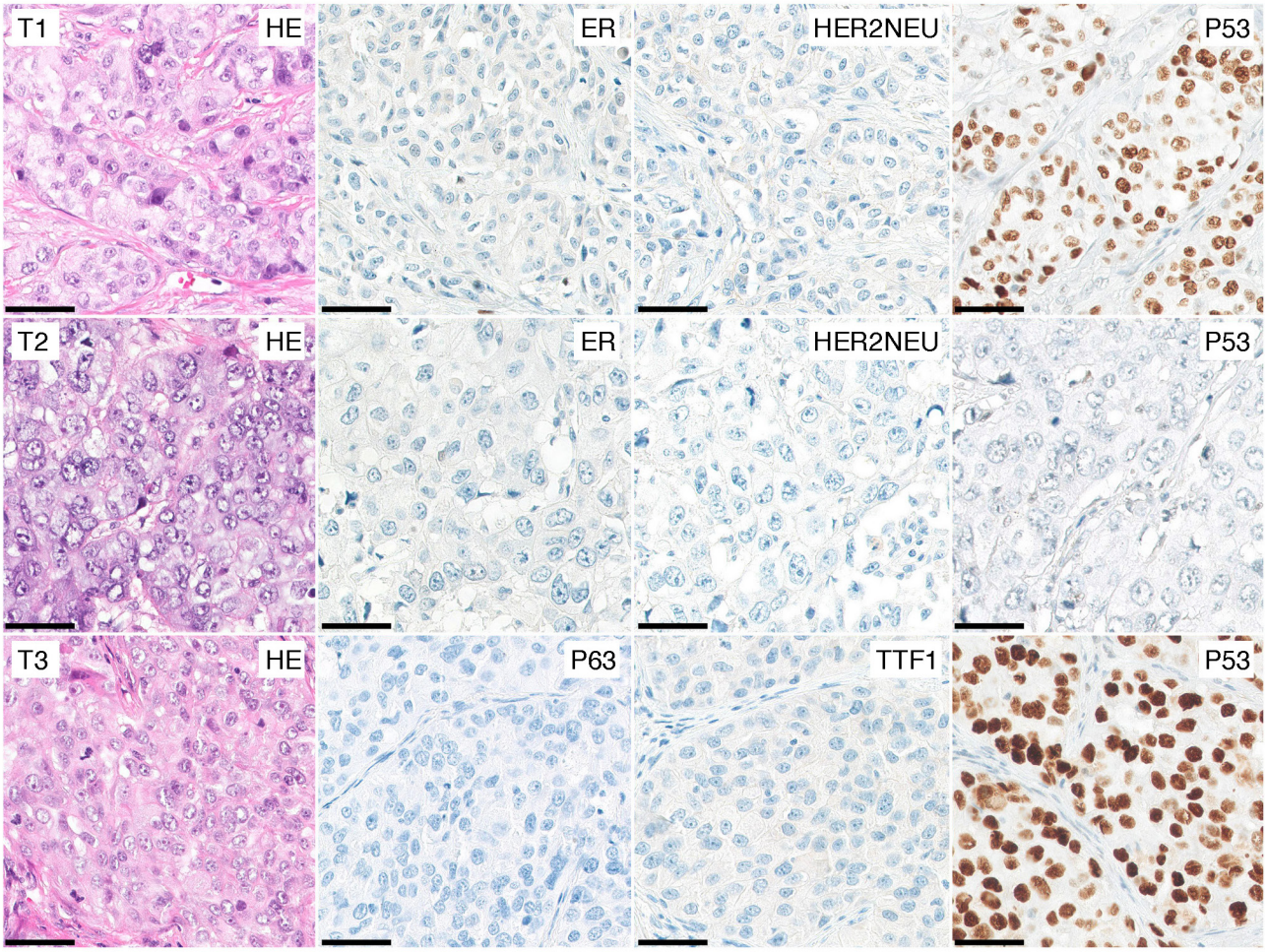

Figure 1. Conventional diagnostics and molecular analysis results for case no. 1, who presented with tumors of the right (T1) and the left (T2) breast, and a tumor in the lung seven years later (T3). (a) Both tumors in the breast (T1 and T2) could be conclusively diagnosed invasive ductal carcinoma of the breast based on haematoxylin and eosin (H&E) stainings only. Additionally, ER and HER2NEU stainings are shown, which were negative in both tumors. Conclusive diagnosis regarding the origin of the non-small cell carcinoma in the lung (T3) based on HE stainings and immunohistochemistry (IHC; P63 and TTF1 both negative) was not possible.
As part of the molecular analysis p53 IHC was performed, showing strong nuclear expression in the tumor cells of T1 and T3, and absent expression in the tumor cells of T2. Scale bars represent 50 µm. (b) Targeted next generation sequencing results of *TP53* exon 6 for DNA isolated from normal and tumor tissues of the patient. Each grey line represents an individual read; only aberrations from the wildtype sequence are indicated. Sequencing results are shown in reverse complement, which means that TCG is actually CGA. T1 and T3 show an identical *PT53* missense mutation (c.646G>A; p.V216M), whereas T2 shows a different *TP53* nonsense mutation (c.637C>T; p.R213*). (c) Loss of heterozygosity (LOH) was analyzed using single nucleotide polymorphisms (SNPs); the variant allele frequencies of 17 SNPs at five different loci (chromosome 8p, *PTEN*, *BRCA2*, *BRCA1* and *SMAD4*) are shown for the three tumor samples. Loss of the reference allele is indicated in red and loss of the variant allele in green; a more intense color (either red or green) indicates a higher tumor percentage. As expected for a *BRCA1* germline mutation carrier, all tumor samples show loss of the same *BRCA1* allele. For all other loci shown, T1 and T3 show corresponding LOH patterns (both tumors show either red or green), whereas T2 shows a different LOH pattern.
Chr: chromosome

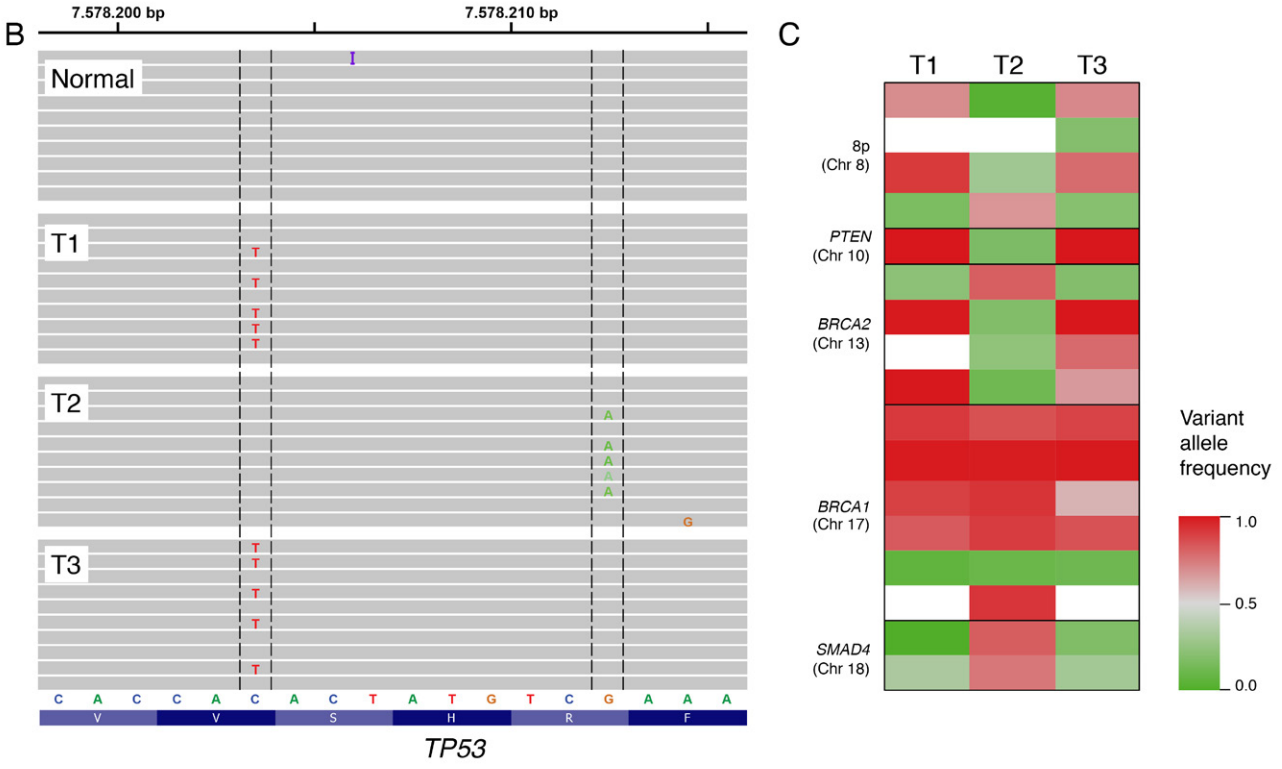

Figure 1. Continued.

Characteristics and outcomes of tumor histology and IHC of cases and controls are outlined in Table 2. Median age at first cancer diagnosis was 41.5 years (range 33-59). Median year of first cancer diagnosis was 1997 (range 1983-2012). Clinical classification of tumor origins was breast cancer + ovarian cancer in 11, breast cancer + other in two, and breast cancer + ovarian cancer + other in one woman.

Molecular analysis

Outcomes of molecular analysis are depicted in Table 2. The FFPE tissues used for DNA isolation were relatively old, ranging from 2 to 32 years old at time of isolation. Six out of 38 (16%) DNA samples isolated from FFPE tumor tissue were of low quality (see Supplementary Table 2 for quality parameters). For six tumors no FFPE tissue was available and DNA was isolated from original routine HE and/or IHC sections or from cytology preparations. Four out of 6 (67%) DNA samples isolated from original sections were of low quality.

In total, 167 tumor-specific variants were detected in the 44 analyzed tumors (Supplementary Table 3). Up to 27 variants were detected in the low quality tumor samples, compared to only one or two variants for good quality tumor samples. Additionally, some multinucleotide changes were incorrectly reported as two or three separate variants. Finally, 48 mutations were either detected in good quality samples or confirmed in low quality samples. In the majority of tumors (n=34, 77%), one mutation was found; seven tumors harbored two mutations. Thirty-nine (81%) of all 48 variants concerned a mutation in the *TP53* gene; in 39/44 tumors (89%) a *TP53* mutation was found. Other variants included *PTEN*, *PIK3CA* and *STK11* mutations in tumors located in the breast; a *CAPZB* mutation in tumors in the uterus and cervix; a *FBXW7* mutation in tumors in the ovary and uterus; and a *BRAF* mutation in a lung lesion (Table 2). Parallel to molecular analysis, p53 IHC was conducted and showed results consistent with molecular outcomes (Table 2). In two low quality samples with aberrant p53 staining, no *TP53* mutation was detected, probably due to insufficient coverage of *TP53* (<100 reads for 8/19 amplicons for control no. 2, T1) or the type of *TP53* mutation

Table 2. Cases and controls: patient characteristics and outcomes of tumor histology, IHC and molecular analysis.

***BRCA1/2+*, age**	**Tumor sites analyzed, timeline (*years*)**			**Histology (H&E) with immunohistochemistry (IHC), if applicable**				**Molecular analysis**				**Agreement molecular analysis with histopathology**
				Conclusive[b]		**Diagnosis[c]**	**Tumor origin[c]**	**p53 IHC[d]**	**Variants by next generation sequencing per tumor**	**Entity[e]**	**Agreement with LOH analysis**	
				H&E	**IHC**							
Case 1 *BRCA1+*, 41 y	0	Right breast[a]	T1	+		Invasise ductal carcinoma	Breast	+	*TP53* c.646G>A; p.V216M	**1**	+	NA
	0	Left breast	T2	+		Invasise ductal carcinoma	Breast	-	*TP53* c.637C>T; p.R213*	**2**		
	7	Lung[a]	T3	-	-	Non-small cell carcinoma	Unknown	+	*TP53* c.646G>A; p.V216M	**1**		
Case 2 *BRCA1+*, 55 y	0	Ovary	T1	+		Serous carcinoma	Ovary	-	*TP53* c.158G>A; p.W53*	**1**	+/-	NA
	6	Breast[a]	T2	-	ND[f]	Adenocarcinoma	Unknown	ND[f]	*TP53* c.158G>A; p.W53*	**1**		
Case 3 *BRCA1+*, 33 y	0	Breast[a]	T1	+		Invasise ductal carcinoma	Breast	ND[f]	*TP53* c.686_687del; p.C229fs*10	**1**	NE	NA
	10	Ascites[a]	T2	-	-	Adenocarcinoma	Unknown	ND[f]	*TP53* c.527G>A; p.C176Y	**2**		
Case 4 *BRCA1+*, 38 y	0	Breast	T1	+		Invasise ductal carcinoma	Breast	+	*TP53* c.318_326delinsAAA; p.S106_F109delinsRN	**1**	+	NA
	3	Retroperitoneal lymph node	T2	-	ND[f]	Large cell carcinoma	Unknown	ND[f]	*TP53* c.514G>T; p.V172F	**2**		
	3	Ovary & uterus	T3	+		Serous carcinoma	Ovary	+	*TP53* c.514G>T; p.V172F	**2**		
Control 1 *BRCA1+*, 38 y	0	Right breast[a]	T1	+		Invasise ductal carcinoma	Breast	-	No mutations	**1**	+	+
	13	Right breast	T2	+		Invasise ductal carcinoma	Breast	+	*TP53* c.722C>T; p.S241F *PTEN* c.176C>G; p.S59*	**2**		
	19	Ovary	T3	-	+	Serous carcinoma	Ovary	+	*TP53* c.400T>G; p.F134V	**3**		
Control 2 *BRCA1+*, 49 y	0	Right breast[a]	T1	+		Invasise ductal carcinoma	Breast	+	No mutations	**1**	NE	+
	2	Adnexa	T2	-	+	Serous carcinoma	Ovary	+	*TP53* c.645T>G; p.S215R	**2**		
	23	Left breast	T3	+		Invasise ductal carcinoma	Breast	+/-	*PIK3CA* c.3140A>G; p.H1047R *STK11* c.484G>A; p.D162N	**3**		
Control 3 *BRCA1+*, 37 y	0	Breast	T1	+		Invasise ductal carcinoma	Breast	+	*TP53* c.817C>T; p.R273C	**1**	+/-	+
	2	Ovary	T2	-	+	Serous carcinoma	Ovary	-	*TP53* c.406C>T; p.Q136*	**2**		
	5	Ovary & uterus (+inguinal & cervical lymph nodes)	T3	-	+	Serous carcinoma	Ovary	-	*TP53* c.406C>T; p.Q136*	**2**		

Table 2. Continued.

BRCA1/2+, age	Tumor sites analyzed, timeline (*years*)			Histology (H&E) with immunohistochemistry (IHC), if applicable				Molecular analysis				Agreement molecular analysis with histopathology
				Conclusive[b]		Diagnosis[c]	Tumor origin[c]	p53 IHC[d]	Variants by next generation sequencing per tumor	Entity[e]	Agreement with LOH analysis	
				H&E	IHC							
Control 4 *BRCA2+*, 60 y	0	Larynx[a]	T1	+		Squamous cell carcinoma	Larynx	-	*TP53* c.375_375+1delinsTT	**1**	+/-	+
	2	Lung	T2	-	+	Adenocarcinoma	Lung	+	*TP53* c.814G>T; p.V272L	**2**		
									BRAF c.1405_1406delinsTT; p.G469L			
	5	Uterus & omentum	T3	+		Serous carcinoma	Ovary	-	*TP53* c.528C>A; p.C176*	**3**		
	9	Breast	T4	+		Invasise ductal carcinoma	Breast	+/-	*PIK3CA* c.3140A>G; p.H1047R	**4**		
Control 5 *BRCA1+*, 37 y	0	Breast	T1	+		Invasise ductal carcinoma	Breast	+	*TP53* c.743G>A; p.R248Q	**1**	+/-	+
	6	Ovary	T2	+		Serous carcinoma	Fallopian tube	-	*TP53* c.395del; p.K132fs*38	**2**		
	7	Omentum	T3	+		Serous carcinoma	Fallopian tube	-	*TP53* c.395del; p.K132fs*38	**2**		
Control 6 *BRCA1+*, 41 y	0	Breast	T1	+		Invasise ductal carcinoma	Breast	+	*TP53* c.743G>A; p.R248Q	**1**	+	+
	0	Cervix	T2	-	+	Adenocarcinoma	Genital tract	-	*TP53* c.721del; p.S241fs*6	**2**		
									CAPZB c.491C>A; p.T164N			
	1	Uterus	T3	-	+	Serous carcinoma	Genital tract	-	*TP53* c.721del; p.S241fs*6	**2**		
									CAPZB c.491C>A; p.T164N			
	1	Omentum	T4	-	+	Serous carcinoma	Genital tract	-	*TP53* c.721del; p.S241fs*6	**2**		
Control 7 *BRCA1+*, 50 y	0	Ovary	T1	+		Serous carcinoma	Ovary	+	*TP53* c.722C>A; p.S241Y	**1**	+	+
	4	Breast	T2	+		Invasise ductal carcinoma	Breast	+/-	No mutations	**2**		
	4	Rectosigmoid	T3	+		Serous carcinoma	Ovary	+	*TP53* c.722C>A; p.S241Y	**1**		
Control 8 *BRCA1+*, 42 y	0	Ovary	T1	+		Serous carcinoma	Ovary	+	*TP53* c.818G>T; p.R273L	**1**	+	+
	0	Omentum	T2	+		Serous carcinoma	Ovary	+	*TP53* c.818G>T; p.R273L	**1**		
	0	Breast	T3	-	+	Invasise ductal carcinoma	Breast	ND[f]	*TP53* c.524G>A; p.R175H	**2**		
	1	Abdominal wall (scar)[a]	T4	+		Serous carcinoma	Ovary	ND[f]	*TP53* c.818G>T; p.R273L	**1**		
	1	Pleural effusion	T5	-	+	Serous carcinoma	Ovary	+	*TP53* c.818G>T; p.R273L	**1**		

Table 2. Continued.

BRCA1/2+, age	Tumor sites analyzed, timeline (*years*)			Histology (H&E) with immunohistochemistry (IHC), if applicable				Molecular analysis				Agreement molecular analysis with histopathology
				Conclusive[b]		Diagnosis[c]	Tumor origin[c]	p53 IHC[d]	Variants by next generation sequencing per tumor	Entity[e]	Agreement with LOH analysis	
				H&E	IHC							
Control 9 *BRCA2+*, 59 y	0	Breast	T1	+		Invasise ductal carcinoma	Breast	-	*TP53* c.327_328dup; p.R110fs*14	**1**	+	+
	2	Ovary[a]	T2	-	+	Serous carcinoma	Ovary	-	*TP53* c.112del; p.Q38fs*6 *FBXW7* c.1347G>C; p.E449D	**2**		
	2	Uterus	T3	-	+	Serous carcinoma	Ovary	-	*TP53* c.112del; p.Q38fs*6 *FBXW7* c.1347G>C; p.E449D	**2**		
Control 10 *BRCA1+*, 45 y	0	Breast	T1	+		Metaplastic carcinoma	Breast	+	*TP53* c.488A>G; p.Y163C	**1**	+	+
	6	Adnexa	T2	+		Serous carcinoma	Ovary	+	*TP53* c.524G>A; p.R175H	**2**		
	6	Rectouterine pouch	T3	+		Serous carcinoma	Ovary	+	*TP53* c.524G>A; p.R175H	**2**		

[a]Low quality sample

[b]Conclusive diagnosis, based on tumor histology (H&E) and IHC, if applicable: yes (+) or no (-)

[c]Based on tumor histology (H&E) and IHC if applicable

[d]Nuclear expression of p53 in tumor cells was scored as either heterogeneous (+-), strong in all tumor cells (+) or absent in all tumor cells (-)

[e]Entity: tumor or tumors most probably of the same origin (clonally identical). 1 is one independent entity, 2 is a second independent entity, etc. Various tumors that form one entity may present advanced disease, cancer relapse, or distant metastases.

[f]No formalin-fixed paraffin-embedded (FFPE) tissue block available or no tissue left in the FFPE tissue block.

H&E: haematoxylin and eosin stained slides; IHC: immunohistochemical analysis; LOH: loss of heterozygosity; NA: not applicable; ND: not done; NE: not evaluable

(possible intronic mutation or homozygous deletion for control no. 1, T1).

As further shown in Table 2, based on the molecular analysis, all tumor localizations analyzed could be classified into one or more entities concerning their origins. Additional LOH analyses of the 143 SNPs at 15 different loci were confirmative of the classifications made in 8/14 patients (Figure 2 + Supplementary Table 4 showing all SNP data). In the group of cases, where conventional histology and IHC were not conclusive, molecular outcomes were decisive for all tumors (see Figure 1 for an example). In the group of controls, all molecular outcomes matched the diagnosis given by conventional histopathological diagnostics.

Figure 2. Loss of heterozygosity (LOH) analysis. Variant allele frequencies (VAF) for single nucleotide polymorphisms (SNPs) at 15 different loci on 11 different chromosomes (indicated on the y-axis) for the tumors samples of all patients analyzed are shown. The VAF for the different SNPs are indicated by different colors. The example (bottom right) shows an A/T SNP, A representing the reference allele and T the variant allele. For any informative SNP without LOH, a VAF of 0.5 is expected (grey). If there is loss of the reference allele, a VAF >0.5 is expected (red). Alternatively, loss of the variant allele would result in a VAF <0.5 (green). A more intense color, either red or green, represents a VAF deviating further from 0.5, indicating a higher tumor percentage. Regardless of the actual nucleotides, green represents the reference allele and red the variant allele for all SNPs. Non-informative SNPs or SNPs with a strand bias or coverage <100 reads are not shown. If multiple tumors of a patient show largely concordant LOH patterns (all tumors show either red or green), this indicates that these tumors are most likely clonally related. Alternatively, differences in the LOH patterns between multiple tumors of one patient indicate multiple primary tumors.

Twelve patients (all patients except control no. 4 and 9) are *BRCA1* mutation carriers. 10/12 patients show a concordant LOH pattern for the *BRCA1* locus in their multiple tumors. Control no. 2 shows an equivocal LOH pattern, which is probably due to the low quality of the data. For case no. 3 only one informative marker is available which does not show clear LOH for T1 (VAF of 0.41). Control no. 4 and 9 are *BRCA2* mutations carriers. Control no. 9 shows a concordant LOH pattern for the *BRCA2* locus for the three analyzed tumor samples. Control no. 4 shows a concordant LOH pattern for samples T2 and T3, a different LOH pattern for sample T1 and no LOH for T4.

Chr: chromosome

Discussion

For 38/42 (90%) *BRCA1/2* mutation carriers with multiple tumor localizations, conventional histopathological analyses (histology, IHC) were sufficient to determine tumor origins. Results obtained by NGS provided decisive information in all four cases with inconclusive results from conventional diagnostics, enabling accurate differentiation between a second primary or metastatic cancer. NGS conducted on 10 control cases with ≥3 tumor localizations, unequivocally showed the same results as obtained by conventional histopathology, and indicate that NGS analysis of multiple tumors within one patient is a versatile procedure to determine clonal relationships between the lesions. NGS analysis can be useful in case of ambiguous histopathology results, or if no FFPE tissue block is available for IHC.

As an illustration, the results of two patients are discussed below. First, case no. 2 comprises ovarian cancer followed by thoracic wall and axillary lymph node metastases three years later. There were no signs of breast cancer, suggesting that the ovarian cancer had metastasized to the thoracic wall and the axilla. After another three years, synchronously with progressive metastatic disease, a small breast cancer was detected. After extensive diagnostic work-up it was concluded that thoracic wall and axillary lesions actually were metastases of this formerly subclinical primary breast cancer and the patient was treated accordingly. However, retrospectively, our findings of identical *TP53* variants in the ovarian cancer and breast cancer strongly suggest that the breast cancer was actually metastatic ovarian cancer. Unfortunately, no suitable material of the thoracic wall and axillary lesions was left for molecular analysis in this study. Since the primary tumor origin determines the therapy of choice for metastatic disease, it is essential to have no doubt about the origin of the metastases. The above-mentioned case is an example of how NGS can be decisive.

Second, control no. 1 comprises two ipsilateral breast cancers with a 13-year interval, both classified as invasive ductal carcinoma by histopathology, and ovarian cancer six years later. Histopathological analysis is not always able to differentiate between local recurrent and second primary breast cancer. The location of the breast cancer may help, but in this case, the first breast cancer was located in the medial upper quadrant while the second breast cancer was located centrally, leaving both options open. Some data suggest that *BRCA1/2* mutation carriers, especially when young (<40 years), show longer intervals to local recurrent breast cancer[23,24]. However, since the prognosis of a second ipsilateral breast cancer occurring <5 years is worse than after >5 years, late-recurring breast cancer are probably more often second primary tumors[25,26] and it is justifiable that they are treated accordingly. It is likely that the recurrent breast cancer after 13 years in this case was a second primary breast cancer. Molecular analysis confirmed that these tumors were two different entities.

LOH -patterns were supportive of the results obtained by variant analysis in more than half of cases and controls (Figure 2). Almost all cases and controls showed corresponding LOH of *BRCA1* or *BRCA2* in all tumors, representing the 'second hit' of the functioning *BRCA* wild-type allele. For *BRCA1* mutation carriers, exceptions were case no. 3 with no clear LOH of *BRCA1* for the breast cancer and control no. 2 with no evaluable LOH results. For *BRCA2* mutations carriers, an exception was control no. 4 with four primary tumors showing loss of one allele of *BRCA2* in the larynx tumor, loss of the other allele in both the lung tumor and the uterus/omentum tumor, and no loss of *BRCA2* in the breast tumor. So far, *BRCA2* mutation carriers are not associated with elevated risk of lung cancer and an increased risk of laryngeal carcinoma seems improbable[27-30]. Additional Sanger sequencing showed loss of the mutated *BRCA2* allele for the tumor located in the larynx and loss of the wild-type allele for the lung lesion and the uterus/omentum tumor localizations (data not shown). The laryngeal carcinoma therefore is most likely a sporadic tumor. Loss of the wild-type *BRCA2* allele in the lung tumor may indicate either sporadic or *BRCA2*-related carcinogenesis. Furthermore, it has been described that LOH causes the second hit in only 80% of *BRCA1*-associated and in 60-70% of *BRCA2*-associated breast cancer[31-33], fitting with the fact that we did not find (clear) LOH in two breast tumors. Possible alternative 'second

hit' mechanisms include mutations and deletions of the wild-type allele. Epigenetic silencing as a second hit, to our knowledge, is rare in germline *BRCA1/2* mutation carriers and therefore not a plausible explanation[31].

The diagnostic panel used in this study covered the exonic regions of the genes *CDKN2A*, *PTEN* and *TP53* almost completely, multiple hotspot mutation sites for 27 genes, and SNPs (Supplementary Table 1). In the majority of cases and controls a conclusive diagnosis concerning tumor site clonality could be made based on different or similar *TP53* variants. A *PTEN* mutation was only found once and none of the tumors harbored *CDKN2A* mutations. Up to 97% of all high grade serous ovarian cancer, typically occurring in *BRCA1/2* germline mutation carriers, harbor somatic *TP53* mutations[12,34]. *TP53* is affected in 16% to 84% of *BRCA1/2*-associated breast cancer, and in up to 97% of *BRCA1*-associated basal-like breast cancer[35,36]. Our finding of *TP53* mutations in 93% of all tumors (39/44 confirmed and 2/44 based on p53 IHC) is in line with the high percentages found in the literature. It suggests that molecular diagnostic workup may simply consist of *TP53* analysis, rather than NGS of an entire panel. However, in two tumors without *TP53* mutations, we found mutations in other genes (*PIK3CA* and *STK11*), providing also a conclusive diagnosis for these tumor localizations. Additionally, LOH analysis was not only confirmative of the classifications made for most of the patients, but was also helpful if 'hotspot' *TP53* mutations were found. An example is control no. 10, for which both T2 and T3 harbor a *TP53 R175H* mutation. Since according to somatic mutation databases this is a common *TP53* mutation these tumors potentially could still be different primary tumors. However, because LOH patterns were identical, we were able to reliably classify these tumors as one entity.

IHC tissue markers were chosen according to institutional protocol depending on clinical and histological differential diagnosis of the tumor origin. Various different IHC markers of breast cancer have been investigated, such as GATA3, GCDFP, mammaglobin and SOX10. Although of potential value for differentiating breast cancer, as yet, their applicability seems limited or has not been validated well enough in triple negative breast cancer [37-39].

A limitation of our study was that 10/44 tumor samples analyzed with NGS were of low quality, mostly due to fixation artefacts or a low amount of starting material, resulting in less reliable variant calling. Variants in low quality samples were therefore confirmed by Sanger sequencing or by a second NGS run. Furthermore, LOH analysis of these samples was difficult, resulting in non-evaluable LOH data in two patients with one or more tumor samples of low quality. Nevertheless, using a combined approach of multiple molecular analyses resulted in reliable classification of the tumors into one or more entities for all patients. Another limitation was that, due to the specific selection criteria, the study sample size was small.

In conclusion, during diagnostic workup of *BRCA1/2*-associated breast cancer and ovarian cancer patients with multiple tumor localizations, analysis of tumor histology and IHC by a specialized pathologist may be sufficiently conclusive in most cases. However when routine pathology is inconclusive, molecular analysis using NGS can reliably determine the relationships between the tumor localizations and as such guide the most appropriate treatment for each individual patient.

Supplementary material on the internet

Supplementary Table 1**.** Next generation sequencing primers.
http://www.niekgeurts.nl/proefschrift/Chapter 6 - Supplementary Table 1.xlsx

Supplementary Table 2**.** All variants detected for the 44 tumors analyzed by next generation sequencing (NGS).
http://www.niekgeurts.nl/proefschrift/Chapter 6 - Supplementary Table 2.xlsx

Supplementary Table 3**.** Quality parameters.
http://www.niekgeurts.nl/proefschrift/Chapter 6 - Supplementary Table 3.xlsx

Supplementary Table 4**.** All single nucleotide polymorphism (SNP) data.
http://www.niekgeurts.nl/proefschrift/Chapter 6 - Supplementary Table 4.xlsx

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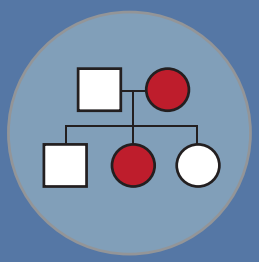

Chapter 7

Identification of Familial Adenomatous Polyposis carriers among children with desmoid tumors

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Abstract

Objective: Desmoid tumors are rare mesenchymal tumors with unpredictable progression and high recurrence risk. They can occur sporadically or in association with Familial Adenomatous Polyposis (FAP), which is caused by germline *APC* mutations. The Wnt/β-catenin pathway has a central role in the pathogenesis of desmoid tumors. These tumors can occur due to either a somatic *CTNNB1* or *APC* mutation but can also be the first manifestation of FAP. Because germline *APC* analysis is not routinely performed in children with desmoid tumors, the diagnosis FAP may escape detection. The aim of this study is to form guidelines for the identification of possible *APC* germline mutation carriers among children with desmoid tumors, based on *CTNNB1* mutation analysis and immunohistochemical analysis (IHC) for β-catenin.

Patients and methods: We performed IHC of β-catenin and mutation analysis of *CTNNB1* and *APC* in 18 pediatric desmoid tumors, diagnosed between 1990 and 2009 in the Erasmus MC, Rotterdam.

Results: In 11 tumors, IHC showed an abnormal nuclear β-catenin accumulation. In this group a *CTNNB1* mutation was detected in seven tumors. In two tumors with an abnormal nuclear β-catenin accumulation and no *CTNNB1* mutation, an *APC* mutation was identified, which appeared to be a germline mutation.

Conclusions: Aberrant staining of β-catenin in pediatric desmoids helps to identify children at risk for FAP. We recommend to screen pediatric desmoid tumors for nuclear localization of β-catenin and consequently for *CTNNB1* mutations. For patients with nuclear β-catenin expression and no *CTNNB1* mutations, *APC* mutation analysis should be offered after genetic counselling.

Introduction

Desmoid tumors (also named aggressive fibromatosis) are rare mesenchymal tumors which can occur sporadically or in association with Familial Adenomatous Polyposis (FAP). These tumors are characterized by unpredictable progression and high recurrence rate and therefore are difficult to treat. The occurrence of desmoid tumors in children is low, with an estimated incidence of 2–4 new diagnoses per million per year[1]. Currently, no general guidelines are available for genetic analysis of pediatric patients with desmoid tumors.

The Wnt/β-catenin signaling pathway is recognized as having a central role in the pathogenesis of desmoid tumors. The β-catenin protein is a key effector of the pathway, affecting cellular decisions such as stem cell maintenance and cell proliferation through modulating the expression of specific target genes. In normal cells, β-catenin is involved both in cell adhesion, when located at the cell membrane, and in transcriptional regulation, when present in the nucleus[2,3]. Several members of the Wnt/β-catenin signaling pathway form an intracellular multiprotein complex, composed of APC, β-catenin, AXIN1, AXIN2 and GSK3b. APC binds to β-catenin at the so-called 15 and 20 amino acid binding sites[2,3]. AXIN1/AXIN2 activates casein kinase I alpha, that catalyses a priming phosphorylation on S45 of β-catenin, thereby providing a signal for glycogen synthase kinase 3 to promote the sequential phosphorylation of T41, S37 and S33, which subsequently induces the degradation of β-catenin thereby preventing its signaling activity.

In several tumor types, this pathway is constitutively activated due to 'loss of function' mutations of the *APC* gene leading to inefficient β-catenin degradation and its intracellular stabilization. In case the *APC* gene is intact, pathogenic *CTNNB1* mutations, encoding β-catenin, are found at the N-terminal phosphorylation sites interfering with its proteolytic degradation[2,3]. Either *APC* or *CTNNB1* mutations can lead to an abnormal intranuclear accumulation of β-catenin. The aberrant β-catenin stabilization is thought to constitutively activate downstream Wnt/β-catenin target genes and trigger a genetic program resulting in tumor formation.

Several studies identified somatic *CTNNB1* mutations in desmoid tumors in a frequency varying between 52% and 87%[4-8], including one study focusing on pediatric desmoid tumors[7]. Somatic *APC* mutations were found in several cases of desmoid fibromatosis, lacking *CTNNB1* mutations[8,9]. In Familial Adenomatous Polyposis (FAP), caused by germline *APC* mutations, 10–15% of the patients are affected by desmoid tumors, representing a more than 800-fold increased risk in comparison with the general population[10,11]. FAP is a hereditary predisposition to develop hundreds to thousands of colorectal polyps ultimately leading to colorectal cancer. Untreated, the risk of colorectal cancer in FAP patients is 100%. The average age when colorectal polyps are detected is 15 years[12]. Colonoscopy, started at the age of 10–12 years, and surgery in adolescence prevent colorectal cancer formation in FAP. When FAP is diagnosed in time, health benefits and increased life expectancy can be achieved.

In children, a desmoid tumor can occur due to either a somatic *CTNNB1* or *APC* mutation, but importantly can also be the first manifestation of FAP. This is of importance for the management of the child, but might also have implications for other (asymptomatic) family members. Currently, no established procedure is available to identify FAP carriers among children with this tumor type. Here, we present an immunohistochemical and mutational analysis on 18 pediatric desmoid tumors, based on which we formulate guidelines to identify possible *APC* germline mutation carriers.

Materials and methods

Patients

Between January 1990 and June 2009, 20 desmoid tumors were operated in 19 patients under the age of 21 years in the Erasmus MC, University Medical Centre, Rotterdam. Ten tumors were localized in the head and neck region, five in the extremities, three in the abdominal wall and two in the back (Table 1). All tumors were classified as deep fibromatoses. The medical records and family history of these patients were analyzed. Tissues were obtained from the initial operative procedure and embedded in paraffin

Table 1. Clinical characteristics of the 19 children with desmoid tumors.

Patient no.	Tumor	Gender	Age at diagnosis (years)	Tumor localization	Family history
1	D1	M	2.4	Mandible	
2	D2	M	8.2	Spina iliaca posterior superior left	
3	D3	F	1.4	Upper extremity	
4	D4	F	4	Neck	
5	D5	F	5	Mandible	
6	D6	F	12.3	Lower extremity	
7	D7	M	1.4	Trunk (back, subcutaneous)	Sister with a fibrous hamartoma of infancy
	D8			Upper extremity	
8	D9	M	5.7	Mandible	
9	D10	F	14.8	Abdominal wall (intraabdominal)	
10	D11	F	15.3	Abdominal wall	This patient and her father diagnosed with FAP
11	D12	M	0.8	Neck	
12	D13	F	0.6	Neck (parotid)	
13	D14	M	0, Congenital	Upper extremity	
14	D15	F	2	Mandible	
15	D16	M	0.8	Trunk (groin)	
16	D17	M	1	Trunk (back, subcutaneous)	
17	D18	F	6	Neck	
18	D19	F	1.3	Earlobe	
19	D20	M	1.5	Parotid gland	Father diagnosed with FAP after diagnosis of the desmoid tumor in the patient

M: male; F: female

after formalin fixation. All tissues were revised by a pathologist and confirmed as being desmoid tumors. Unfortunately, two samples (D14 and D18) were not suitable for molecular analysis.

Immunohistochemistry (IHC)

Immunohistochemical analysis for β-catenin (1:100 dilution of clone 14, BD Transduction Laboratories) was performed basically as previously described[13]. All sections were evaluated under a light microscope after Mayer hematoxylin counterstaining.

DNA isolation

Normal and tumor DNA was extracted from Formalin-Fixed, Paraffin-Embedded (FFPE) tissue fragments using proteinase K and 5% Chelex 100 resin, as previously described[14]. For the identification of germline *APC* mutations, DNA isolated from peripheral blood cells was used. Isolation of this DNA was performed according to standard procedures.

CTNNB1 mutation analysis

Sequence analysis of *CTNNB1* exon 3, encoding the mutational cluster region (MCR) of β-catenin was performed on tumor DNA as previously described[14]. To test for complete or partial deletions of *CTNNB1* exon 3, a PCR was performed using primers flanking exon 3.

APC mutation analysis

As the MCR of *APC* in desmoid tumors is located in exon 15 between codons 1324 and 1567, this region was chosen for sequence analysis[8,15,16]. The *APC* MCR was amplified in four overlapping fragments according to standard procedures.

For the identification of germline *APC* mutations, DNA isolated from peripheral blood cells was used. All coding exons and intron–exon boundaries of *APC* were sequenced according to standard procedures. In addition, Multiplex ligation-dependent probe amplification (MLPA) analysis was performed using the SALSA MLPA kit P043 (MRC

Holland, Amsterdam, The Netherlands) as previously described[17].

LOH

Loss of heterozygosity (LOH) analysis of the *APC* locus was performed using DNA isolated from normal and tumor tissues. Three microsatellite markers (D5S433, D5S656, D5S421), mapping to chromosome 5q21.2-5q22.2 were selected. PCR amplification was performed as described above for *CTNNB1* mutation analysis with FAM-labelled primers. The FAM-labelled PCR fragments were run on an ABI 3130xl genetic analyzer (Applied Biosystems) and data were analyzed with Genemarker version 1.8 software (SoftGenetics, State College, PA). Peak heights of the alleles were compared between normal and tumor DNA samples. If one of the alleles showed relative loss in the tumor sample this was considered to be due to LOH (scored manually).

Sequences of all primers are available on request.

Results

Eighteen desmoid tumors from 17 patients could be included in the study (Table 1 and 2). Patient 7 showed two localizations of desmoid tumors (D7 and D8). His sister had a fibrous hamartoma of infancy. In patient 10 there was a prior family history of FAP. The father of patient 19 was known with polyposis. After diagnosis of a desmoid tumor in patient 19 at 1.5 years of age, we could identify a germline *APC* mutation in the father as well as in the child during subsequent counselling. The youngest age at diagnosis was 0 years (a congenital desmoid tumor) and the oldest patient was 15 years. The latter was the patient from the previously known FAP family. The mean age was 4.4 years. The male: female ratio was almost equal (1:1.1).

Immunohistochemistry

Immunohistochemistry succeeded in all 18 tumors. Eleven tumors (61%) showed strong nuclear staining for β-catenin (Table 2 and Figure 1a and c). No nuclear

Table 2. Results of immunohistochemical analysis (IHC) and mutation analyses of *CTNNB1* and *APC*.

Sample	IHC β-catenin	*CTNNB1* analysis	*APC* analysis	Effect of *APC* mutation	LOH of *APC*
D1	MS	wt	wt		No
D2	IS	c.121A>G; p.T41A	–		–
D3	LIS	wt	wt		No
D4	LIS	–	–		–
D5	IS	c.104T>G; p.I35S	wt		No
D6	IS	c.121A>G; p.T41A	wt		No
D7	CS	wt	wt		–
D8	IS	wt	wt		–
D9	LIS	–	–		–
D10	IS	c.134C>T; p.S45F	wt		–
D11	IS	wt	c.4216C>T; p.Q1406*	Nonsense (heterozygous)	No
D12	IS	c.134C>T; p.S45F	wt		No
D13	IS	c.122C>T; p.T41I	wt		No
D15	IS	c.133T>C; p.S45P	wt		No
D16	LIS, LCS	wt	wt		No
D17	CS	wt	–		–
D19	IS	wt	wt		No
D20	IS	wt	c.4348C>T p.R1450*	Nonsense (hemizygous)	Yes

In samples D14 and D18, IHC of β-catenin and DNA-isolation did not succeed due to low cellular density and the poor quality of FFPE stored tumor samples (no sequences obtained).

Wt: wild type; '–': no informative result; IS: intranuclear staining; MS: membranous staining; LIS: light intranuclear staining; CS: cytoplasmic staining; LCS: light cytoplasmic staining

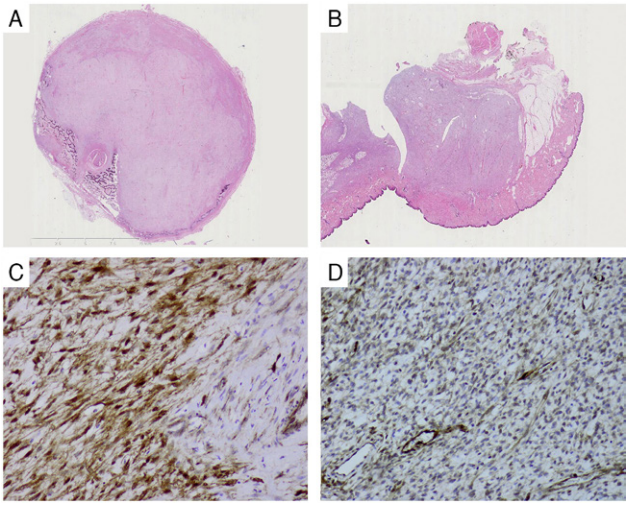

Figure 1. Microscopy and immunohistochemistry. (a) and (b), hematoxylin and eosin (HE) stained section of two desmoid tumors. The same tumors were used for β-catenin immunohistochemistry: (c) note the strong nuclear accumulation of β-catenin in the tumor cells whereas the accompanying normal cells are negative; (d) note the absence of nuclear staining and the presence of membranous staining of β-catenin in the tumor cells. The blood vessels show a normal positive membranous and cytoplasmic β-catenin staining.

expression of β-catenin was present in normal cells in these tissues. In seven other tumors at most a light nuclear, membrane or cytoplasm staining was present (Figure 1b and d).

CTNNB1 mutation analysis

Sequence analysis of exon 3 of *CTNNB1* succeeded in 16/18 tumors. No data were obtained from the remaining two samples due to poor quality DNA within these samples. In 7/16 tumors (44%) point mutations were found (Table 2 and Figure 2a). Six mutations were situated in the expected positions (residues S45 and T41). One mutation was detected at position 35 (p.I35S), which has been described previously[18,19]. Analysis for exon 3 deletions revealed no obvious deletions, but small deletions could have been missed, due to the relative large size of the residual product and poor quality of DNA extracted from FFPE material. All oncogenic *CTNNB1* mutations were identified in tumors showing a strong nuclear accumulation of β-catenin (7/11).

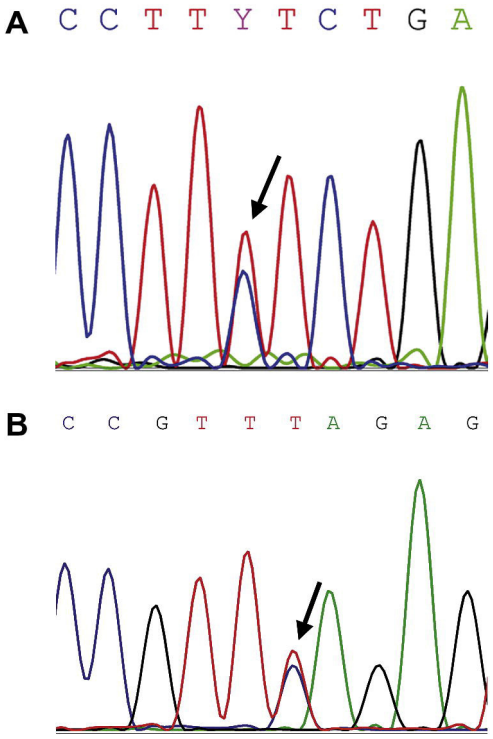

Figure 2. (a) Sequence analysis of *CTNNB1* (D12) showing heterozygosity for the p.S45F mutation; (b) sequence analysis of *APC* (D11) showing heterozygosity for the p.Q1406X mutation.

APC mutation analysis

Mutation analysis of the *APC* MCR succeeded in 14/ 18 tumors. An *APC* mutation was found in two samples (14%), both causing a premature stop codon (Table 2 and Figure 2b). Both mutations were also found in the germline DNA of the patients. Ten samples harbored the heterozygous c.4479G>A change, known as polymorphism (data not shown). No *APC* mutation was detected in the *CTNNB1* mutation positive samples. Both desmoid tumors in which we identified the *APC* mutation also showed a strong accumulation of nuclear β-catenin (2/11).

LOH analysis

To further study the possible involvement of *APC* in the development of the desmoid tumors, LOH analysis of the *APC* locus was performed in all tumors. Only one tumor, i.e. D20 in which we had previously identified the p.R1450X germline mutation, showed LOH of the *APC* locus (Figure 3).

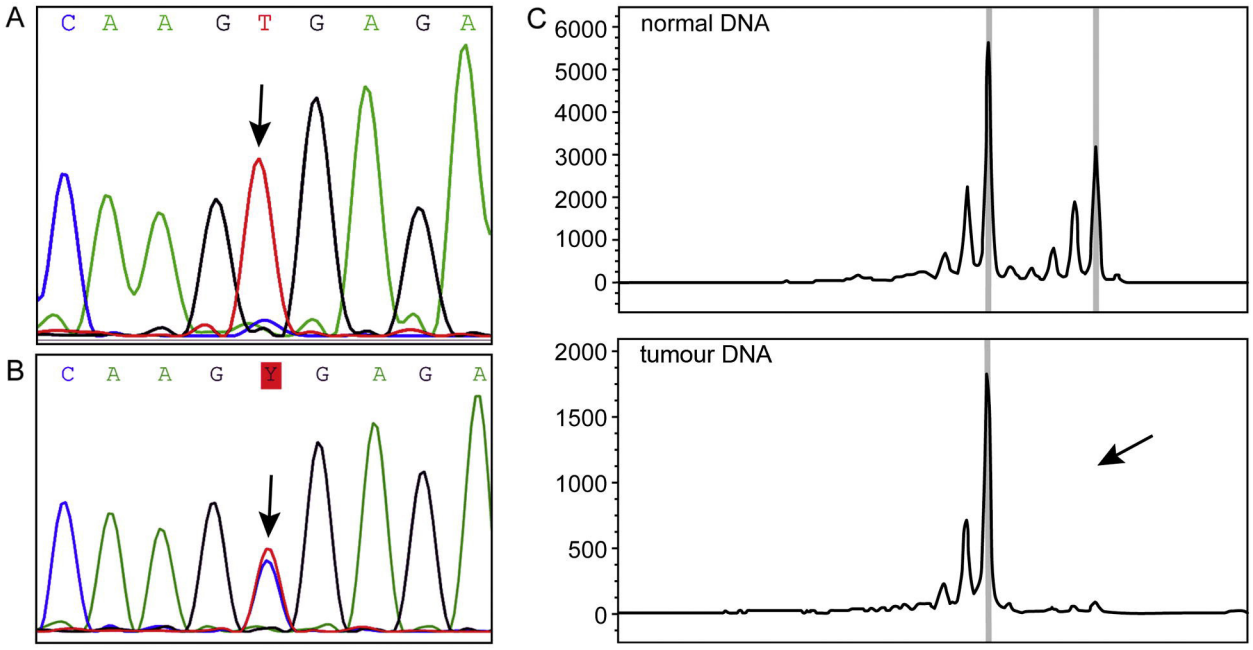

Figure 3. (a) Sequence analysis of *APC* in the tumor (D20), showing hemizygosity for the c.4348C>T p.R1450X mutation; (b) Identification of the germline *APC* mutation in patient no 19, showing heterozygosity for the c.4348C>T p.R1450X mutation; (c) Loss of heterozygosity (LOH) analysis in tumor D20, showing loss of heterozygosity of the *APC* locus (marker D5S656). The arrow indicates the loss of one allele in the tumor DNA.

Discussion

We analyzed 18 pediatric desmoid tumors for aberrations in the Wnt/β-catenin signaling pathway. Immunohistochemical analysis for β-catenin indicated that deregulation of this pathway was involved in the development of 11 tumors, of which two harbored point mutations in the *APC* gene (D11, D20, Table 2). These were confirmed as being a germline *APC* mutation. Patient 10 was a member of a known FAP family. The father of patient 19 was known with polyposis. During the following genetic counselling, we identified both father and son as being a carrier of the p.R1450X germline *APC* mutation. We identified loss of the remaining wild type allele (LOH of *APC*) in the desmoid tumor of patient 19. This is in line with Knudson's two-hit hypothesis[20-22], further supporting that the germline *APC* mutation underlies formation of this tumor.

The results of the molecular and immunohistochemical analyses of the desmoid tumors of patient 10 and 19 indicate that these analyses can lead to the identification of FAP carriers.

Mutations in *CTNNB1* and *APC* have been shown to occur in a mutually exclusive manner in all tumor types studied so far. As germline oncogenic *CTNNB1* mutations are not compatible with adult life, the identification of such a mutation provides direct evidence of the sporadic nature of the tumor. *CTNNB1* mutations are more common than *APC* mutations in adult desmoid tumors, suggesting that most desmoid tumors are of sporadic origin[4-8]. Recently, Bo et al.[7] showed that *CTNNB1* is also frequently mutated in pediatric desmoid tumors (25/32), but FAP patients were excluded in their study. We observed a somewhat lower overall mutation frequency than previous reports, which may be explained by the limited number of cases in our cohort. However, the less frequent involvement of Wnt signaling pathway in pediatric desmoid tumors has been previously described as well[23]. Also in our set, no simultaneous *APC* mutations were detected in the tumors with a somatic *CTNNB1* mutation, and as such these tumors can be considered as sporadic tumors.

Interestingly, we observed two samples with intranuclear staining for β-catenin in which neither *CTNNB1* nor *APC* mutations were detected. However, we still do suspect an involvement of the Wnt/β-catenin signaling pathway in these samples. An *APC*-

mutation localized outside the analyzed region cannot be excluded. Alternatively, these samples could contain an alteration of other Wnt-related genes, such as AXIN1 or AXIN2, although mutations in these genes have not been reported in desmoid tumors previously. Also epigenetic alterations may play an important role in these tumors. Recently, Okpanyi et al.[24] showed activation of the Wnt/ β-catenin pathway in pediatric germ cell tumors due to *APC* promoter methylation and LOH of *APC*. In colorectal cancer an epigenetic inactivation of secreted frizzled related proteins (SFRPs), which normally suppresses Wnt signaling, has been suggested to contribute to colon cancer formation[25]. As the epigenetic mechanisms in desmoids tumors are not well understood, it should be investigated in the future.

In four desmoid tumors, neither nuclear β-catenin staining nor *CTNNB1* or *APC* mutations were detected. At this moment we have no evidence of Wnt/β-catenin pathway involvement in these tumors, and the aetiology of these tumors remains unclear. Previous studies described a lack of *CTNNB1* and *APC* gene mutations in superficial fibromatoses[26]. However, since all tumors in our cohort were classified as deep fibromatoses, this feature represents an unlikely explanation for the lack of mutation in these mutation-negative tumors. Interestingly, patient 7 developed two desmoid tumors with both presence and absence of intranuclear staining of β-catenin in different tumors. His sister had a fibrous hamartoma of infancy. Previously it has been reported that Wnt/β-catenin pathway plays an important role in desmoid formation, but does not appear to play a role in the pathogenesis of other myofibroblastic lesions in children[27]. Pathological diagnosis of fibroblastic lesions can be challenging. As such, it cannot be entirely excluded that some of the immuno-negative lesions might not be desmoid tumors, although central review by an experienced pathologist has been performed.

Despite the limited size of our cohort, our data support current ideas about the molecular background in desmoid tumors and illustrate that a combination of IHC of β-catenin and the mutation analysis of *CTNNB1* and *APC* can help to identify *APC* carriers among the children with desmoid tumors. This may be especially valuable in de novo *APC* mutation carriers, with a negative family history for FAP. It is known that approximately 20–25% of individuals with FAP have a de novo *APC* mutation[28]. Unfortunately, not in all our FFPE samples sufficient DNA quality could be obtained, most likely due to the well-known problems associated with over-fixation and long-term storage in paraffin. To this aim, we recommend that fresh frozen tissue of the desmoid sample is stored specifically for DNA isolation, in addition to the FFPE sample. The latter is still required for a proper histological evaluation and for β-catenin IHC, as it is not possible to detect nuclear β-catenin in frozen sections[29].

In conclusion, children with desmoid tumors can be carriers of a germline *APC* mutation. Therefore, we recommend to analyze the lesions for nuclear staining of β-catenin by IHC to identify the desmoid tumors with an underlying defect in β-catenin signaling. If consequently pathogenic *CTNNB1* mutations are detected, this makes an increased risk of FAP in the patient unlikely. If, however, *CTNNB1* screening turns out negative, genetic counselling of such children and their parents is warranted and germline *APC* analysis should be offered. These recommendations should be evaluated by future studies in larger cohorts.

Acknowledgements

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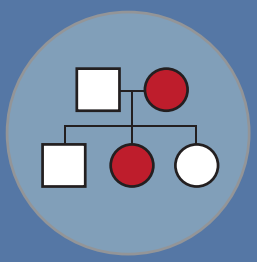

Chapter 8

SHORT REPORT

Pitfalls in molecular analysis for mismatch repair deficiency in a family with biallelic *PMS2* germline mutations

Celine HM Leenen, Willemina RR Geurts-Giele, Hendrikus J Dubbink, Roel E Reddingius, Ans MW van den Ouweland, Carli MJ Tops, Heleen M van de Klift, Ernst J Kuipers, Monique E van Leerdam, Winand NM Dinjens, Anja Wagner

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Abstract

Heterozygous germline mutations in the mismatch repair (MMR) genes *MLH1, MSH2, MSH6* and *PMS2* cause Lynch syndrome. Biallelic mutations in the MMR genes are associated with a childhood cancer syndrome [constitutional mismatch repair-deficiency (CMMR-D)]. This is predominantly characterized by hematological malignancies and tumors of the bowel and brain, often associated with signs of neurofibromatosis type 1 (NF1). Diagnostic strategies for selection of patients for MMR gene analysis include analysis of microsatellite instability (MSI) and immunohistochemical (IHC) analysis of MMR proteins in tumor tissue. We report the clinical characterization and molecular analyses of tumor specimens from a family with biallelic *PMS2* germline mutations. This illustrates the pitfalls of present molecular screening strategies. Tumor tissues of five family members were analyzed for MSI and IHC. MSI was observed in only one of the analyzed tissues. However, IHC analysis of brain tumor tissue of the index patient and his sister showed absence of PMS2 expression, and germline mutation analyses showed biallelic mutations in *PMS2:* p.Ser46Ile and p.Pro246fs. The same heterozygous mutations were confirmed in the father and mother, respectively. These data support the conclusion that in case of a clinical phenotype of CMMR-D, it is advisable to routinely combine MSI analysis with IHC analysis for the expression of MMR proteins. With inconclusive or conflicting results, germline mutation analysis of the MMR genes should be considered after thorough counselling of the patients and/or their relatives.

Introduction

Heterozygous germline mutations in mismatch repair (MMR) genes *MLH1*, *MSH2*, *MSH6* and *PMS2* cause Lynch syndrome[1-5]. Carriers of heterozygous MMR gene mutations are at high risk for developing colorectal carcinomas (CRCs) and extracolonic neoplasias such as endometrial, small bowel, ureter, renal pelvis, stomach, ovarian and brain tumors. In Lynch syndrome carriers, these malignancies usually develop during the fourth and fifth decade of life. Biallelic mutations in MMR genes lead to a childhood cancer syndrome. This is predominantly characterized by hematological malignancies, brain tumors and gastrointestinal tumors in early childhood. Carriers of biallelic MMR gene mutations often show signs of neurofibromatosis type 1 (NF1), mainly café au lait (CAL) spots. This childhood cancer syndrome is often referred to as constitutional mismatch repair-deficiency (CMMR-D). To our knowledge, a total of 107 cases of children with CMMR-D have been reported in the literature[6-10].

Diagnostic strategies for fast selection of patients with an MMR gene defect suspected for Lynch syndrome include analysis of MSI and immunohistochemical (IHC) analysis of tumor tissue for expression of MMR proteins[11-13]. However, the sensitivity of molecular tests in tumor tissue of patients with CMMR-D is unclear. MSI and absent MMR protein staining have been described in gastrointestinal tumors of patients with CMMR-D[9]. In contrast, tumor tissue of most reported CMMR-D patients with brain tumors did not show MSI[8,14].

Here, we report a family with childhood brain tumors and early-onset colorectal cancer with biallelic germline mutations in the *PMS2* gene that underscores pitfalls of the present molecular screening strategy.

Case report

Family data

At age 7, the index patient was diagnosed with an anaplastic glial brain tumor (Figure 1: pedigree, individual IV.2). His older sister (individual IV.1) had died from a primitive neuroectodermal brain tumor (PNET) at 4 years of age. Both children had multiple large CAL spots (Figure 2) and the index patient showed freckling. The younger sister of the index patient (individual IV.3) showed one CAL spot. Both non-consanguineous parents were of Dutch origin and showed no signs of neurofibromatosis type I (NFI). At the time of counseling, the family history of both parents was not suggestive of Lynch syndrome. One maternal uncle had been diagnosed with colorectal cancer (individual II.5) at age 62. The parents of the index patient declined endoscopic screening. However, within 2 years of the diagnosis of the brain tumor of our index patient, the father was diagnosed with colorectal cancer at age 43 (pT4N2M1, Dukes stage D). The paternal mother (individual II.2) was diagnosed with colorectal cancer during the same period of time at age 84. The mother of our index patient then underwent surveillance colonoscopy and one adenoma with low-grade dysplasia was removed.

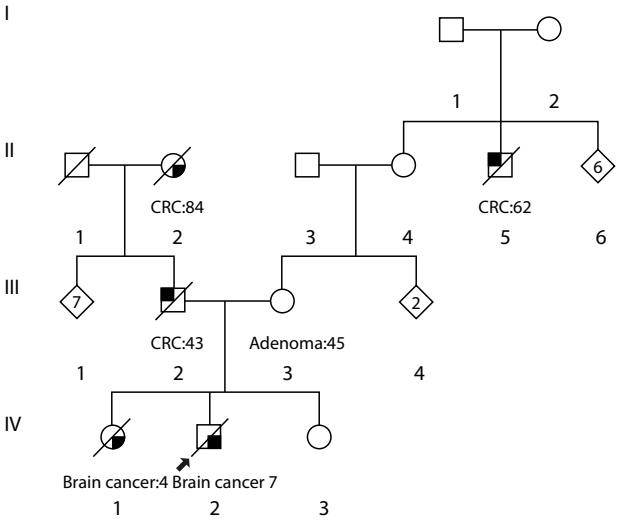

Figure 1. Pedigree of the reported family. Patient details on malignancy, adenomas and age of diagnosis in years are given. → index patient; □ male; ○ female; ◇ males and females; ◪ CRC, coloretal cancer; ◪ brain cancer; / deceased.

MSI and IHC analyses

Tissues of five family members including CRC tissues from the father and his mother, the colonic adenoma from the mother and brain tumor specimens from the index case and his sister were analyzed for MSI

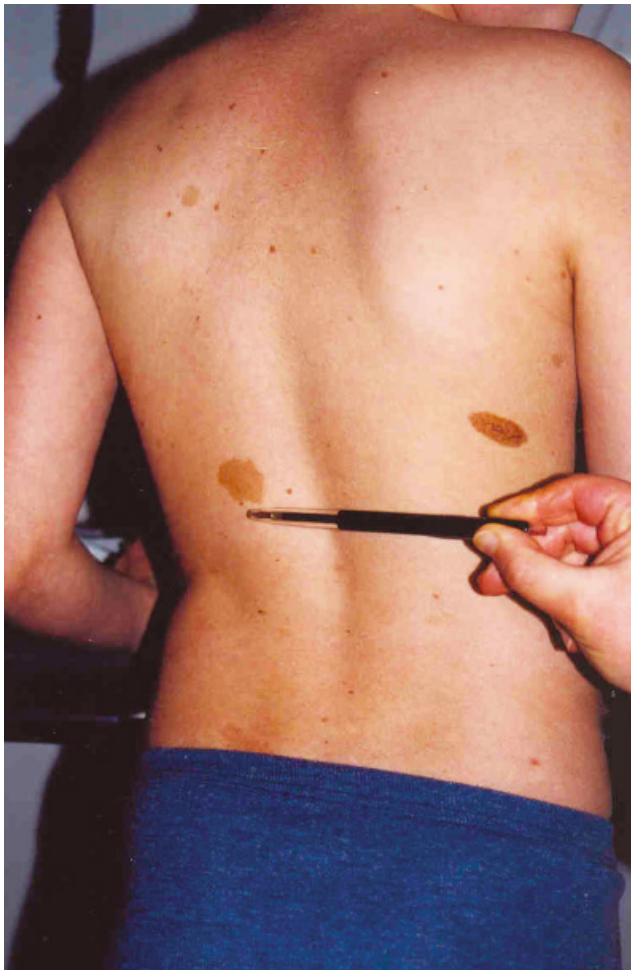

Figure 2. Café au lait spots of the index patient.

and IHC aberrations (Table 1). MSI analysis was performed on DNA retrieved from paraffin-embedded tumor tissues, using five mononucleotide repeat MSI markers (Promega pentaplex) as previously described[14,15]. As controls, normal leukocyte DNA from the index patient, DNA from paraffin-embedded normal tissue from the father (III.2), DNA from paraffin embedded normal tissue from the grandmother (II.2), and unrelated normal DNA were used. The MSI marker profiles of all these normal DNA samples (three family members, one unrelated normal DNA) were identical, demonstrating the absence of MSI in normal tissues. IHC analysis was performed for four MMR proteins: MLH1, MSH2, MSH6 and PMS2, according to the standard procedure[15].

The brain tumor of the index patient showed an MSI pattern with additional fragments of increased size of markers NR-21 and BAT-26. Surprisingly, microsatellite stability (MSS) was observed, in the brain tumor of the sister of the index patient (Figure 3a). IHC analysis of brain tumor tissues from both children showed absence of PMS2 expression in the tumor and normal cells. Tumor specimens from all other family members were MSS and showed normal expression of the MMR proteins in the tumor and normal tissue.

Germline mutation analysis

Mutation analysis of the *NF1* gene was performed in the index patient but a mutation could not be identified. Mutation analysis in a blood sample of the index patient identified the compound heterozygous mutations, p.Pro246fs and p.Ser46IIe.

Table 1. Summary of results of the molecular and IHC analyses of tissues from the studied family.

Case	Malignancy	Age at diagnosis (years)	Skin lesions	NF1 gene mutation	Analysis of MSI	IHC	LOH analysis *PMS2*	*PMS2* gene mutation
II.2	Adenocarcinoma of the rectum	84	ND	ND	MSS	Normal	ND	None
III.2	Adenocarcinoma of the transverse colon	43	None	ND	MSS	Normal	no LOH	Heterozygous p.Ser46IIe
III.3	One adenoma (low-grade dysplasia)	45	None	ND	MSS	Normal	ND	Heterozygous p.Pro246fs
IV.1	PNET	4	CAL spots >6, hemangioma leg	ND	MSS	PMS2 absent	ND	Compound heterozygous Pro246fs, p.Ser46IIe
IV.2	Anaplastic glial brain tumor	7	CAL spots >6, axillary melanotic freckling	None	MSI-H	PMS2 absent	ND	Compound heterozygous Pro246fs, p.Ser46IIe

CAL: café au lait; IHC: immunohistochemistry of MLH1, MSH2, MSH6 and PMS2; LOH: loss of heterozygosity analysis by sequencing; ND: not determined; MSI: microsatellite instability; MSI-H: microsatellite instability-high; MSS: microsatellite stable; PNET: primitive neuroectodermal brain tumor

Both mutations were also found in DNA derived from the brain tissue from his sister. No mutation analysis was performed in the younger sister of the index patient. The heterozygous mutations p.Ser46Ile and p.Pro246fs were confirmed in the father and mother, respectively, indicating the compound heterozygous pattern in the index patient and his sister. The paternal grandmother appeared not to carry the p.Ser46Ile mutation, as present in the father of the index patient.

In both the index patient and his father, no germline mutation was detected in the *MLH1*, *MSH2* and *MSH6* gene.

Additional molecular analysis: loss of heterozygosity (LOH)

To assess whether the tumor from the father of the index patient was caused by the *PMS2* germline mutation, LOH analysis was performed (Figure 3b). For that purpose, DNA extracted from normal and tumor tissue of the father was sequenced, according to a previously described method[14]. No LOH of the *PMS2* locus was found, while the tumor percentage was high enough to detect LOH, which was indicated by the presence of LOH at the *TP53* and *APC* loci (Figure 3c).

Discussion

The above-mentioned family displays a CMMR-D phenotype in the presence of compound heterozygous *PMS2* mutations (p.Ser46Ile and p.Pro246fs). MSI was only found in the brain tumor of the *PMS2* compound heterozygous index patient. The brain tumor of his compound heterozygous sister, as well as the CRCs of the father and his mother and the colorectal adenoma of the mother, were MSS. IHC analysis showed absence of PMS2 staining in both the brain tumor and normal tissue of the index patient and his sister, but not in the analyzed CRCs of their father and grandmother.

PMS2 is considered a tumor suppressor gene[5]. In tumors of carriers of a heterozygous *PMS2* mutation, MSI and absence of IHC staining of PMS2 can be expected as a result of the loss of the wild-type allele. In case of a biallelic germline mutation, MSI and especially absence of PMS2 expression can be expected already in normal tissue, as well as in tumor tissue.

Both parents of the index patients were found to carry a heterozygous *PMS2* mutation. The p.Pro246fs mutation of the mother (individual III.3) is a previously described pathogenic frameshift mutation[16]. The p.Ser46Ile missense germline mutation of the father (individual III.2) has been found in seven cases in a cohort of 400 selected Dutch patients suspected to have an MMR gene defect. In contrast, this mutation was not detected in 927 controls (unpublished data of the Department of Human and Clinical Genetics, LUMC). Also, the amino acid involved in this mutation, is positioned in a highly conserved small helix domain (codon 35–48) and in addition serine and isoleucine have very different physical and chemical properties. In the literature, there is a clear overrepresentation of p.Ser46Ile in patients with PMS2 negative tumors[17-21]. These findings support the pathogenicity of this mutation.

Surprisingly, no MSI and IHC aberrations were found in the CRC of the father. Eight additional CRCs in heterozygous carriers of the p.Ser46Ile mutation have been reported (Table 2). Unfortunately, only data on the MSI status of the tumor tissues of three of these eight patients were available, all displaying MSI. Absence of PMS2 expression was found in all described tumors in contrast to our observations in the CRC of the father. Because additionally no LOH of the *PMS2* locus was detected in the tumor of the father, a role of *PMS2* in the development of the early-onset CRC of the father cannot be demonstrated at the moment. It is possible that other colorectal cancer susceptibility genes are involved. As the tumor tissue of the father's CRC was found to be MSS, it is unlikely, however, that this concerns the other MMR genes. Also, germline mutation analyses of *MLH1*, *MSH2* and *MSH6* revealed no aberrations in the father. Nevertheless, other unknown susceptibility genes cannot be excluded. In view of this, first-degree relatives of the father, who test negative for the familial *PMS2* mutation, should in our opinion still be offered colorectal surveillance.

The results of MSI and IHC analysis of the tissue of the paternal grandmother are in agreement with analysis of sporadic colorectal cancer. This

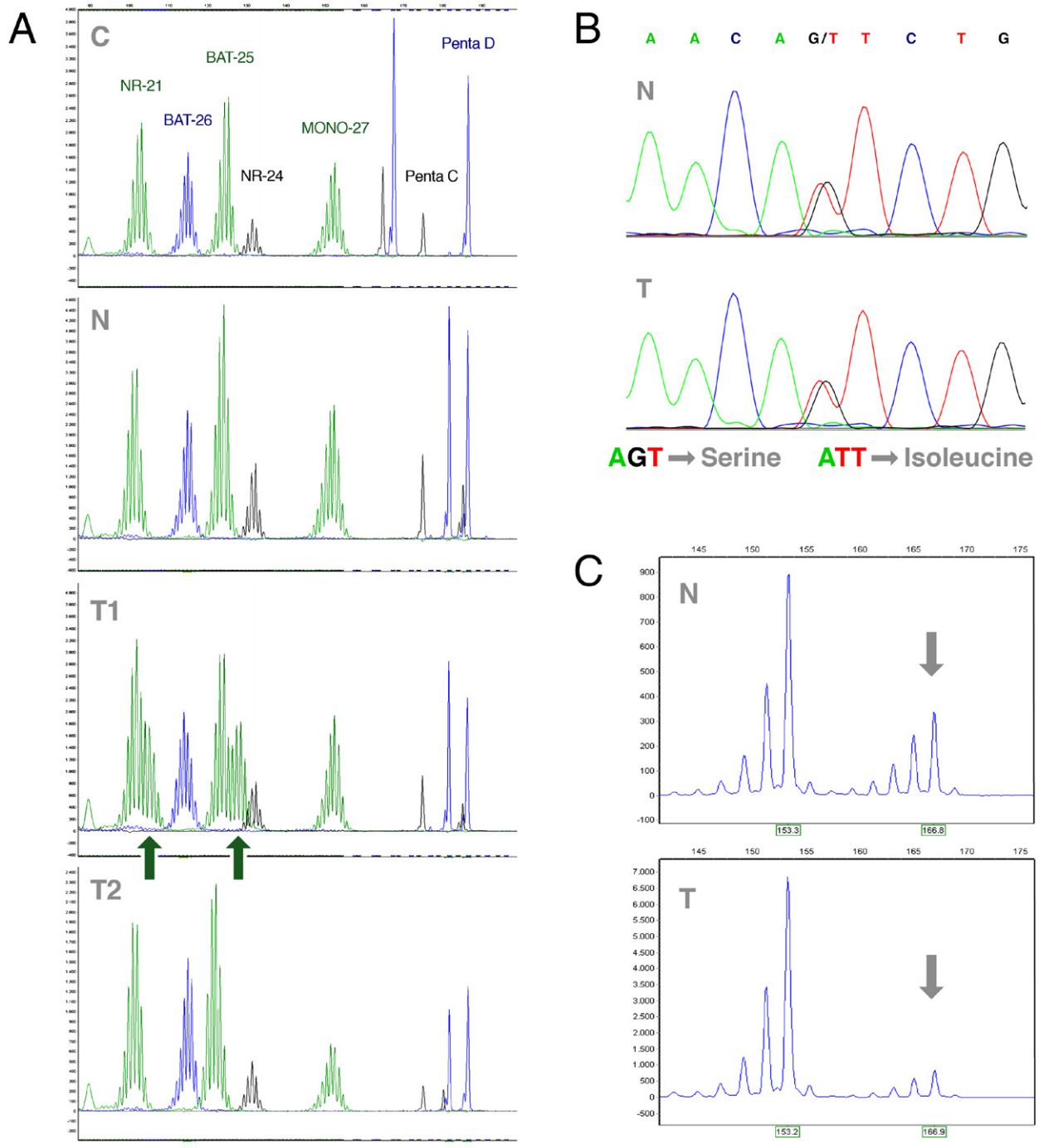

Figure 3. Microsatellite instability (MSI) analysis of the brain tumors of the index patient and his sister (individual IV.2 and IV.1) and loss of heterozygosity (LOH) analysis of the father of the index patient (individual III.2). (a) Promega pentaplex MSI results of unrelated normal control DNA (C), index patient's normal leukocyte DNA (N), index patient's brain tumor DNA (T1) and his sister's brain tumor DNA (T2). MSI (markers NR-21 and BAT-25, instability indicated by arrows) is only observed in the tumor of the index patient and not in his sister's tumor. (b) Sequencing results of exon 2 of the *PMS2* gene, showing the heterozygous p.Ser46Ile (c.137G>T) mutation in normal tissue (N) and colorectal carcinomas (CRC) (T) of the father. No LOH is detected in tumor tissue (T). (c) Result of LOH analysis with a dinucleotide polymorphic microsatellite at the *TP53* locus in normal tissue (N) and CRC (T) of the father. Arrows indicate the relative loss of the larger allele in the CRC compared to the normal DNA.

Table 2. Genetic and clinical summary of nine reported cases (including our case) of patients with CRC and the heterozygous *PMS2* mutation: c.137G>T; p.Ser46Ile.

Patient	Case	Malignancy	Age at diagnosis of malignancy (years)	Analysis of MSI	IHC	References
1	1	CRC: cecum	32	NA	PMS2 absent	Senter et al., 2008[20]
2	2	CRC: cecum	47	NA	PMS2 absent	Senter et al., 2008[20]
3	3	CRC: sigmoid	44	NA	PMS2 absent	Senter et al., 2008[20]
4	5	CRC: transverse	43	NA	PMS2 absent	Senter et al., 2008[20]
5	6	CRC: sigmoid	62	NA	PMS2 absent	Senter et al., 2008[20]
6	Patient 1	CRC	31	MSI-H	PMS2 absent	Nakagawa et al., 2004[19]
7	66603/current report: III.2	CRC	43	MSS	Normal	Van der Klift et al., 2010[21]
8	74028	CRC	70	MSI-H	PMS2 absent	Van der Klift et al., 2010[21]
9	74055	CRC	54	MSI-H	PMS2 absent	Van der Klift et al., 2010[21]

CRC: colorectal carcinoma; IHC: immunohistochemistry; NR: not reported; MSI: microsatellite instability; MSI-H: microsatellite instability-high; MSS: microsatellite stable

finding is in concordance with her not being a carrier of the familial *PMS2* mutation.

The index patient and his sister inherited both *PMS2* germline mutations from their parents, explaining their CMMR-D phenotype. However, MSI was found in only one of the two brain tumors. In gastrointestinal tumors, MSI analysis seems to be a reliable tool to diagnose MMR deficiency. In the literature, results of molecular analyses in 21 patients with gastrointestinal malignancies and biallelic MMR gene mutations have been reported. Nineteen patients were diagnosed with CRC and two patients with duodenal cancer. In all tumors, MSI was detected. Additional IHC analysis showed absence of immunostaining of the corresponding affected MMR proteins in 19/21 analyzed gastrointestinal tumors[8,17,18,20,22-34]. In addition to the gastrointestinal patients, 43 patients (mean 8 years, range 4–17, 88% male) with biallelic MMR gene mutations and brain cancer have been reported. In 8 of these 43 cases, brain tumor specimens were analyzed for MSI and in 5 of these cases IHC analysis of MMR proteins was performed (Table 3; [8,14,17,25,35-38]). Germline mutation analysis showed one patient with *MLH1*, one with *MSH2*, four with *MSH6* and two with *PMS2* mutations. In six of the analyzed eight cases, no MSI was found in brain tumor tissue. A hypothesis to explain the lack of MSI in brain tumors from germline biallelic *PMS2* mutant patients is that in brain tissue a PMS2 deficiency could lead to tumorigenesis through a different mechanism than the MMR pathway[8,14,17]. Also, the extent and pattern of MSI may differ between CRCs and brain tumors, making the MSI analysis that is routinely used for CRC less reliable for brain tumors[8,14].

IHC analysis showed the absence of immunostaining of PMS2 in the brain tumor cells as well as in normal cells in the specimens of our index case and his sister. This is in accordance with the absence of expression of the affected MMR protein in all five investigated brain tumors of germline biallelic mutant MMR gene patients described in the literature[8,14,17,35,36]. From the literature and our own data, it can be concluded that MMR and IHC analysis may be more sensitive than MSI analysis to detect MMR deficiency in brain tumors.

The third child in this family (IV.3) is also at risk of being a heterozygous or compound heterozygous carrier of the familial *PMS2* mutations. Because single CAL spots are a frequent finding in the general population and this child is 8 years past the age of onset of the brain tumors in her siblings, we estimate her risk for CMMR-D to be lower than the theoretical 25%. However, her risk is not excluded. No guidelines are available yet for the surveillance of children at risk for CMMR-D. In this family, we think regular clinical surveillance by a pediatric oncologist including colonoscopy and possibly brain magnetic

Table 3. Results of analysis for MSI and IHC analysis of patients with brain cancer from families with biallelic MMR mutations.

Family	Case	Gene	Malignancy	Age at diagnosis of malignancy (years)	Signs of NF1	Analysis of MSI	IHC	References
1	Patient1	*PMS2*	Glioblastoma, colonic adenomas, NHL of the rectum	4	CAL spots	MSI-H	NA	Hamilton et al., 1995[25]
2	IV.2	*MSH2*	Glioblastoma	4	NR	MSS	NA	Bougeard et al., 2003[35]
3	III.1	*PMS2*	Giant cell glioblastoma, duodenal cancer, colonic adenomas	17	CAL spots	MSS (glioblastoma), MSI-H (duodenal cancer)	PMS2 absent in glioblastoma. MSH6 and PMS2 absent in duodenal tumor tissue	Agostini et al., 2005[17]
4	V.4	*MSH6*	Oligodendroglioma, rectosigmoid cancer	10	CAL spots	MSS	MSH6 absent in CRC, MSH6 present in oligodendroglioma	Menko et al., 2004[8]
5	IV.3	*MSH6*	Glioblastoma multiforme	8	CAL spots, axillary freckling	MSI-H	NA	Hegde et al., 2005[37]
6	Patient1	*MSH6*	Astrocytoma	9	CAL spots, axillary freckling, IgA deficiency	NA	MSH6 absent	Ostergaard et al., 2005[38]
7	I	*MLH1*	Glioblastoma, Wilms tumor	4	CAL spots	MSS	MLH/PMS2 absent in brain tissue	Poley et al., 2007[14]
8	I.2	*MSH6*	Glioblastoma multiforme	9	Hyper and hypopigmentation skin	MSS	MSH6 absent, MSH2 expression reduced	Etzler et al., 2008[36]

CAL: café au lait; IgA: immunoglobulin A; IHC: immunohistochemistry; NA: not available; MMR: mismatch repair; MSI: microsatellite instability; MSI-H: microsatellite instability-high; MSS: microsatellite stable; NHL: non-Hodgkin's lymphoma

resonance imaging (MRI) can be considered. Because of behavioral and psychological problems of the third child, she and her mother declined genetic testing for the *PMS2* mutations and surveillance at the moment.

In conclusion, the results of molecular analyses in this family display the diagnostic challenges in *PMS2*-mutation families. In case of a clinical phenotype of CMMR-D, it is recommended to routinely combine MSI analysis with IHC analysis for the expression of MMR proteins. With inconclusive or conflicting results, mutation analysis of the MMR genes should be considered after thorough counselling of the patients and their relatives.

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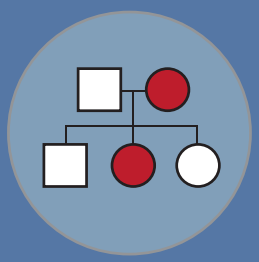

Chapter 9

Somatic aberrations of mismatch repair genes as a cause of microsatellite-unstable cancers

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Abstract

Lynch syndrome (LS) is caused by germline mutations in mismatch repair (MMR) genes, resulting in microsatellite-unstable tumors. Approximately 35% of suspected LS (sLS) patients test negative for germline MMR gene mutations, hampering conclusive LS diagnosis. The aim of this study was to investigate somatic MMR gene aberrations in microsatellite-unstable colorectal and endometrial cancers of sLS patients negative for germline MMR gene mutations. Suspected LS cases were selected from a retrospective Clinical Genetics Department diagnostic cohort and from a prospective multicenter population-based study on LS in The Netherlands. In total, microsatellite-unstable tumors of 40 sLS patients (male/female 20/20, median age 57 years) were screened for somatic MMR gene mutations by next-generation sequencing. In addition, loss of heterozygosity (LOH) of the affected MMR genes in these tumors as well as in 68 LS-associated tumors and 27 microsatellite-unstable tumors with *MLH1* promoter hypermethylation was studied. Of the sLS cases, 5/40 (13%) tumors had two pathogenic somatic mutations and 16/40 (40%) tumors had a (likely) pathogenic mutation and LOH. Overall,
LOH of the affected MMR gene locus was observed in 24/39 (62%) tumors with informative LOH markers. Of the LS cases and the tumors with *MLH1* promoter hypermethylation, 39/61 (64%) and 2/21 (10%) tumors, respectively, demonstrated LOH. Half of microsatellite-unstable tumors of sLS patients without germline MMR gene mutations had two (likely) deleterious somatic MMR gene aberrations, indicating their sporadic origin. Therefore, we advocate adding somatic mutation and LOH analysis of the MMR genes to the molecular diagnostic workflow of LS.

Introduction

Lynch syndrome (LS) is an autosomal dominant hereditary condition that predisposes to various types of cancer and accounts for about 3% of all colorectal cancers (CRCs) and about 2% of all endometrial cancers (ECs)[1,2]. The increased risk for malignant lesions in LS is due to an inactivating germline mutation in one of four mismatch repair (MMR) genes, *MLH1*, *MSH2*, *MSH6* and *PMS2*, or a germline deletion in *EPCAM*[3,4]. The final diagnosis of LS is based on the identification of the germline mutation in one of these genes[5].

MMR genes are classical tumor suppressor genes and bi-allelic inactivation results in tumorigenesis. The tumors of LS patients are characterized by a microsatellite instability (MSI) phenotype and absence of expression of one or more MMR proteins, both indicating DNAMMR deficiency. As a result of the LS testing algorithm[6], patients are indicated as suspected of LS (sLS) or non-suspected of LS, after which germline testing of the affected MMR gene(s), as indicated by immunohistochemistry (IHC), is performed in the sLS cases. Germline testing leads to identification of a MMR gene mutation or of a variant of unknown significance (VUS) in about 65% of the sLS patients, as was shown in a prospective multicenter population-based study in The Netherlands, in which all consecutive CRC and EC patients≤70 years were screened for LS[7,8]. The lack of identification of mutations in the remaining 35% severely hampers conclusive diagnosis (LS or not LS) for these patients and their relatives. An existing germline mutation could have been missed by germline analysis or they could have a sporadic tumor caused by bi-allelic somatic MMR gene inactivation. The prevalence of somatic mutations in the *MLH1*, *MSH2* and *MSH6* genes in sporadic CRC is 16%, 10% and 6%, respectively[9,10]; however, not all of the tumors included in these analyses showed MSI. More recently, somatic aberrations of the *MLH1* and *MSH2* genes were studied in CRCs and ECs with MSI, but negative for both MMR germline mutations and promoter hypermethylation[11,12]. Sourrouille et al.[11] performed mutation analysis of 18 CRCs and detected two somatic mutations in each of four tumors. Mensenkamp et al.[12] combined mutation and LOH analysis in 25 CRCs or ECs and identified two somatic hits in 13 tumors. Both studies concluded that these double somatic hits indicated bi-allelic somatic inactivation and sporadic occurrence of the tumors.

Reliable LS diagnosis is important for both patients with malignancies and their healthy relatives at risk of carrying a MMR gene germline mutation, as surveillance and preventive options can provide substantial health benefits in the case of a pathogenic MMR germline mutation[13-16]. In addition, the exclusion of LS in patients suspected of LS can also lead to health benefits, since these patients and their relatives may be released from further surveillance, additional genetic testing and emotional distress. The aim of the present study was to improve LS diagnostics by the determination of somatic MMR gene aberrations in microsatellite-unstable tumors of sLS patients tested negative for germline MMR gene mutations.

Materials and methods

Patient selection and DNA isolation

Patient selection is described in Figure 1; unexplained tumors from sLS patients were included in the study. sLS patients were defined as patients: (a) with microsatellite-unstable CRC, EC or ovarian cancer; (b) without *MLH1* promoter hypermethylation when *MLH1* was the affected MMR gene as indicated by IHC; and (c) tested negative for germline mutations and VUS in the affected MMR gene (mutation analysis of entire genes, including analysis of large intragenic deletions) and negative for *EPCAM* deletions. If blood was not available as the source of constitutional DNA because the patient was deceased at the time of germline mutation analysis, one or more first-degree relatives were analyzed.

A retrospective series of 22 tumors (including one adenoma) of sLS patients were screened for MMR gene aberrations; these patients or their relatives were counselled at the Clinical Genetics Department of Erasmus MC, University Medical Centre, Rotterdam, during 2000–2012. Furthermore, 18 tumors of sLS patients that were previously involved in a prospective multicenter population-

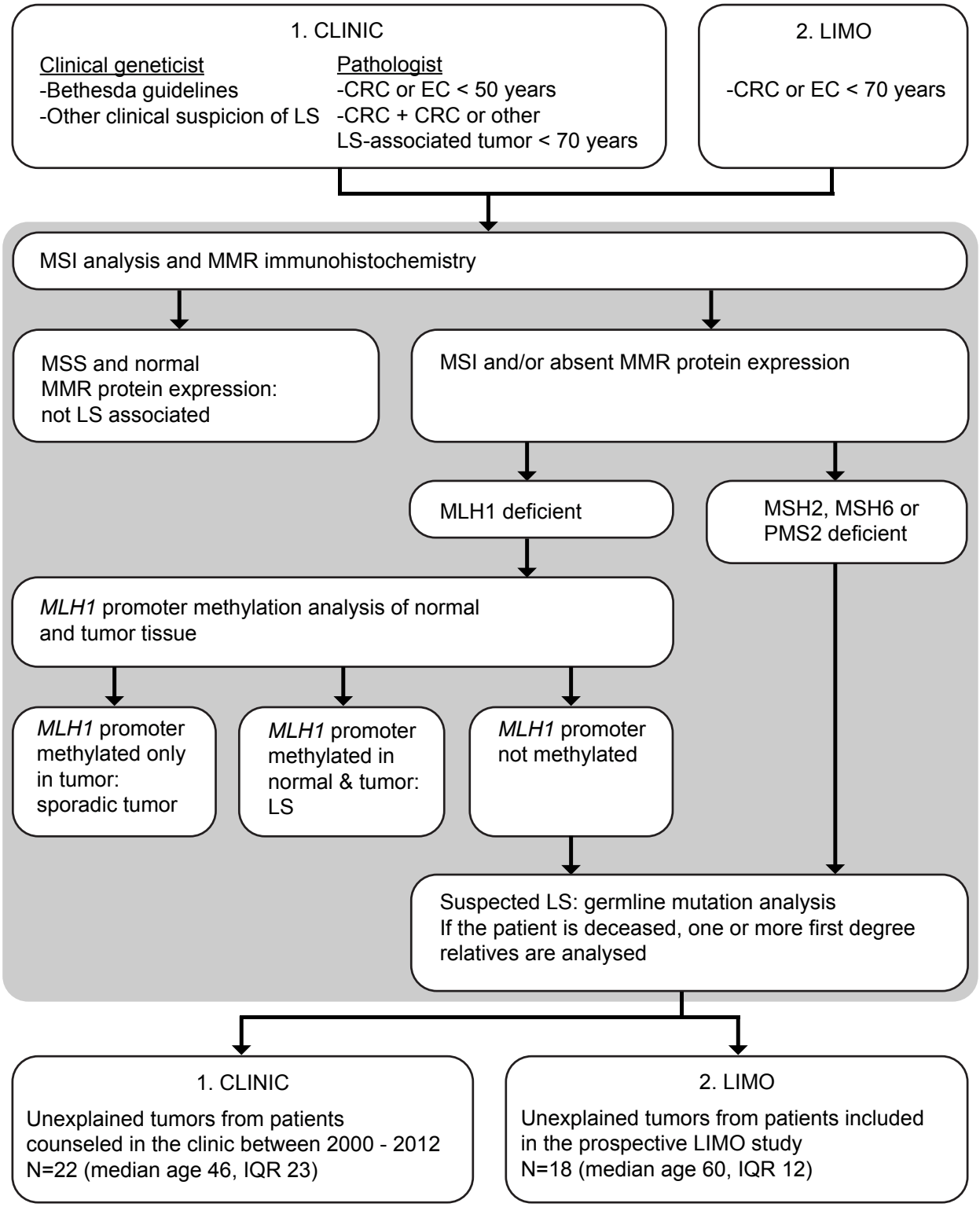

Figure 1. Patient selection flowchart. All unexplained tumors (without germline mutations) from a retrospective series of suspected Lynch syndrome (sLS) patients counselled at the Clinical Genetics Department of Erasmus MC during 2000–2012 (1. CLINIC) as well as from sLS patients previously involved in a prospective, multicenter, population-based study in The Netherlands (2. LIMO) were included.

CRC: colorectal cancer; EC: endometrial cancer; LS: Lynch syndrome; MMR: mismatch repair; MSI: microsatellite instability; MSS: microsatellite-stable; n: number of patients

based study in The Netherlands (LIMO) were included[7,8]. As controls, 68 tumors of LS patients with an identified pathogenic MMR gene germline mutation (male/female 35/33; median age 50 years; IQR 14) and 27 sporadic tumors with *MLH1* promoter hypermethylation (male/female 9/18; median age 64 years; IQR 8) were analyzed. Of all 135 cases, formalin-fixed and paraffin-embedded (FFPE) normal and tumor tissues were manually microdissected from five to ten hematoxylin-stained sections. DNA was extracted using proteinase K and 5% Chelex 100 resin, as previously described[6].

MSI analysis, MMR protein IHC and *MLH1*, *MSH2* and *MSH6* promoter hypermethylation assay

These analyses were performed as previously described[6] (for additional details, see Supplementary Methods).

LOH analysis and copy number detection of the MMR genes

LOH analysis was performed for the affected MMR gene (for sLS patients, as indicated by IHC) using the SNaPshot multiplex kit (Applied Biosystems, Foster City, CA, USA) on normal and tumor DNA, as previously described[17]. Single and multiplex PCR assays were designed to detect six to nine single-nucleotide polymorphisms (SNPs) in or adjacent to each of the MMR genes (Figure 2) (see Supplementary Methods). Classification of SNP results per gene was as follows: LOH, at least one SNP with LOH, no SNP with retention of heterozygosity (ROH); ROH, at least one SNP with ROH, no SNP with LOH; partial LOH, both SNP(s) with LOH and ROH; NI, all SNPs were non-informative (homozygous). To establish the copy number of the affected MMR gene, fluorescence in situ hybridization (FISH) was performed, using a commercial probe to detect *MSH2* and custom-made probes to detect *MLH1*, *MSH6* or *PMS2* (all Kreatech, Amsterdam, The Netherlands), according to standard protocols. Control probes targeting the centromere or a locus on the opposite chromosomal arm were included for each gene.

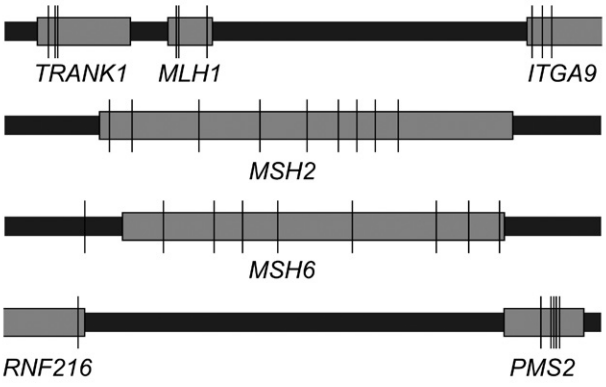

Figure 2. Locations of the SNPs used to screen for LOH of the MMR genes in four multiplex assays. Vertical black lines represent the positions of the SNPs targeted by SNaPshot probes.

Mutation analysis of the MMR genes and *BRAF*

All tumor samples of sLS patients were screened for somatic mutations of *MLH1*, *MSH2*, *MSH6* and *PMS2*, using the ion torrent personal genome machine (PGM) with the supplier's materials and protocols (Life Technologies, Carlsbad, CA, USA). A custom primer panel targeting the open reading frame including the exon–intron boundaries of the MMR genes was designed using Ion AmpliSeq Designer 1.2. This panel consisted of 150 amplicons covering 100%, 92%, 97% and 79% of *MLH1*, *MSH2*, *MSH6* and *PMS2*, respectively. Mean amplicon size was 155 (range 124–174) base pairs (bp). All variants in the coding regions and the splice sites were reported, excluding synonymous single-nucleotide variants and known bona fide SNPs. All variants detected with the PGM were confirmed by Sanger sequencing in tumor and normal DNA, as previously described[18]. For four MLH1-deficient tumors (sLS-1, sLS-2, sLS-9 and sLS-10), conventional Sanger sequencing of the exonic regions of *MLH1* was performed instead of PGM analysis. All previously identified germline mutations in LS patients were confirmed in normal and tumor tissue if possible. Additionally, all tumor samples were screened for *BRAF* mutations by Sanger sequencing and with mutation-specific PCR for *BRAF* V600E and V600K, using FAM-labelled primers. Details of the PGM and Sanger sequencing analyses and data processing are provided in the Supplementary Methods.

Predicting pathogenicity for somatic MMR variants

Frame-shift, nonsense and splice site mutations were

assumed to be pathogenic. For all missense variants and in-frame deletions, InSIGHT classification[19,20], multiple in silico tools and a literature search were used to predict pathogenicity (for more details, see Supplementary Methods). Finally, all variants were classified as: 1, benign; 2, likely not pathogenic; 3, uncertain; 4, likely pathogenic; or 5, definitely pathogenic.

Results

All results for sLS patients, confirmed LS patients and patients with sporadic tumors are shown in Supplementary Table 1.

Somatic MMR aberrations in sLS patients

The tumors of 40 sLS patients negative for MMR gene germline mutations were screened for somatic MMR mutations and for LOH of the affected MMR gene, as indicated by IHC (Table 1, Figure 3). This led to the detection of 49 somatic MMR gene variants, 31 in *MLH1*, 11 in *MSH2*, six in *MSH6* and one in *PMS2*. Details about PGM coverage and the classification of missense variants and in-frame deletions are shown in Supplementary Tables 1 and 2, respectively. Two (5%) of the 40 patients, both deceased and with first-degree relatives negative for germline MMR mutations, showed a pathogenic mutation in both tumor and normal tissues. For all other patients, DNA from normal tissue showed no aberrations. Twenty-one out of 40 (53%) tumors showed either two pathogenic mutations (5) or one (likely) pathogenic mutation and LOH (16) (Figure 4). In 12 of the tumors with a mutation and LOH, Sanger sequencing confirmed loss of the wild-type allele. Five of the 40 (13%) tumors showed a VUS combined with a pathogenic mutation or LOH. In 9/40 (23%) tumors, only one somatic aberration was detected and 1/40 (3%) tumors showed only a likely benign variant. Two (5%) of 40 tumors showed no aberrations, including the tumor from patient sLS-38, for which no mutation analysis results were available. In total, two VUS and four likely benign variants, but no pathogenic mutations, were detected in non-affected MMR genes, as indicated by IHC.

LOH analysis

All tumors were screened for LOH of the affected MMR gene (for sLS patients, as indicated by IHC) by SNaPshot analysis and/or Sanger sequencing. Finally, 39/40 tumors from sLS patients, 61/68 tumors from LS patients and 21/27 sporadic tumors showed evaluable and informative results. Of those, 24/39 (62%) tumors of sLS patients, 39/61 (64%) LS-associated tumors and 2/21 (10%) sporadic tumors

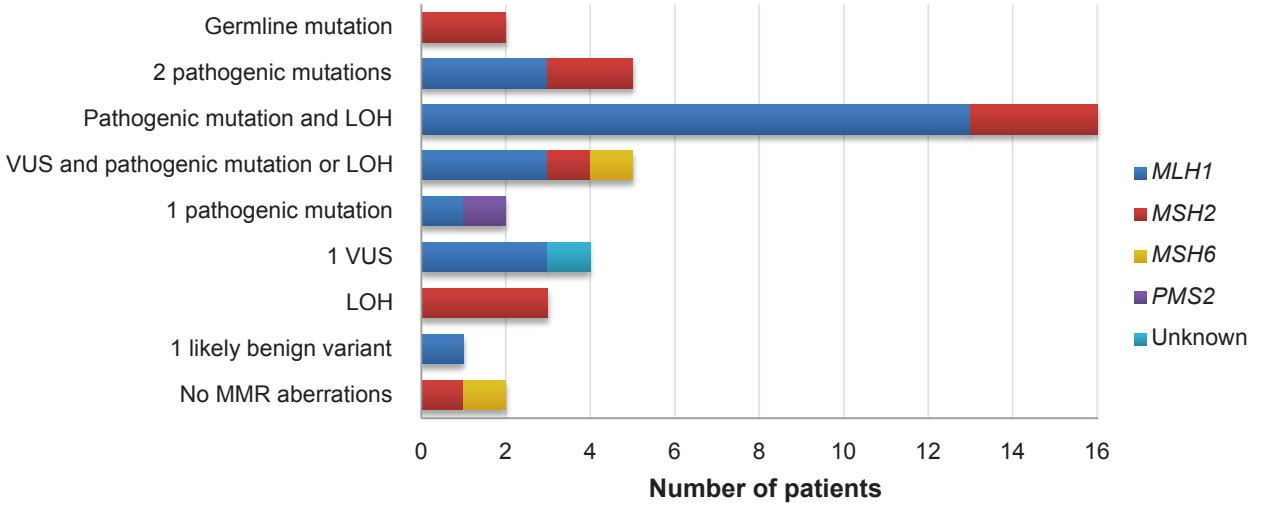

Figure 3. Somatic aberrations for the tumors of 40 suspected Lynch syndrome patients. The different colors indicate the deficient mismatch repair gene, as indicated by immunohistochemistry.
LOH: loss of heterozygosity; VUS: variant of unknown significance

Table 1. Somatic aberrations for suspected LS patients.

Patient	Cohort	M/F	Age[a]	Tumor	Germline analysis	Somatic variants	Class (1-5)	% of variant reads with PGM (variant reads/ total reads)	LOH/ROH Sanger	LOH/ROH SNaPshot	Copy number FISH	*BRAF*	Conclusion somatic MMR aberrations
MLH1													
sLS-1	C	F	46	EC	A	c.37delG; p.E13fs*3	5	PGM results ND	ROH	ROH	2	ND	2 pathogenic mutations
						c.1816_1817delGG; p.G606fs*2	5		ROH				
sLS-2	C	M	61	CRC	A	c.790+1G>A	5	PGM results NE	ROH	ROH	2	WT	2 pathogenic mutations
						c.1852_1854delAAG; p.K618del	5		ROH				
sLS-3	C	M	33	CRC	A	c.2059delC; p.R687fs	5	82% (437/536)	LOH	LOH	NE	c.1781A>G; p.D594G	Pathogenic mutation and LOH
sLS-4	C	F	46	CRC	A	c.298C>T; p.R100*	5	86% (730/852)	LOH	LOH	2	WT	Pathogenic mutation and LOH
sLS-5	C	M	46	CRC	B	c.1276delC; p.Q426fs	5	89% (1782/2000)	LOH	LOH	2	WT	Pathogenic mutation and LOH
sLS-6	C	F	59	CRC	A	c.350C>T; p.T117M	5	60% (838/1402)	NE	LOH (1 marker)	2	c.1799T>A; p.V600E	Pathogenic mutation and LOH
sLS-7	C	F	68	CRC	A	c.146T>C; p.V49A	3	44% (2562/5773)	ROH	LOH	2	WT	Pathogenic mutation and LOH
						c.2001delC; p.D667fs	5	90% (751/838)	LOH				
sLS-8	C	F	73	CRC	B	c.1A>T; p.M1L	3	81% (520/645)	ROH	LOH	2	WT	Pathogenic mutation and LOH
						c.1852_1854delAAG; p.K618del	5	22% (449/2000)	ROH				
sLS-9	C	M	39	Villous adenoma	A	c.453+1G>A	5	PGM results ND	ROH	ROH	2	ND	VUS and pathogenic mutation
						c.2270A>T, p.*757L	3		ROH				
sLS-10	C	F	65	CRC	A	c.793C>G; p.R265G	3	PGM results ND	LOH	LOH	2	WT	VUS and LOH
sLS-11	C	F	40	CRC	A	c.638delT; p.V213fs	5	52% (518/1005)	ROH	ROH	2	WT	1 pathogenic mutation
sLS-12	C	F	51	CRC	B	c.1652A>C; p.N551T	3	44% (382/868)	NE	NE	2	NE	1 VUS
sLS-13	C	M	45	CRC	A	c.1270G>A; p.A424T (only PGM)	2	72% (146/202)	ND	ROH (1 marker)	2	c.1799T>A; p.V600E	1 likely benign variant
sLS-14	L	F	65	CRC	B	c.445C>T; p.Q149*	5	33% (366/1123)	ROH	NI	ND	WT	2 pathogenic mutations
						c.676C>T; p.R226*	5	34% (400/1180)	ROH				
						MSH6: c.2914A>T; p.I972F	2	31% (217/689)	ROH				
sLS-15	L	M	64	CRC	A	c.678-1G>A	5	89% (271/304)	LOH	NE	2	WT	Pathogenic mutation and LOH
sLS-16	L	M	70	CRC	A	c.638delT; p.V213fs	5	83% (1592/1917)	LOH	LOH	2	c.1801A>G; p.K601E	Pathogenic mutation and LOH
sLS-17	L	F	59	CRC	B	c.1608delT; p.P536fs	5	80% (991/1242)	LOH	LOH	2	WT	Pathogenic mutation and LOH

Table 1. Continued.

Patient	Cohort	M/F	Age[a]	Tumor	Germline analysis	Somatic variants	Class (1-5)	% of variant reads with PGM (variant reads/ total reads)	LOH/ROH Sanger	LOH/ROH SNaPshot	Copy number FISH	*BRAF*	Conclusion somatic MMR aberrations
sLS-18	L	M	61	CRC	A	c.199G>A; p.G67R	5	72% (2831/3907)	LOH	LOH (1 marker)	NE	WT	Pathogenic mutation and LOH
sLS-19	L	F	62	CRC	A	c.350C>T; p.T117M	5	81% (130/161)	NE	LOH	2	WT	Pathogenic mutation and LOH
sLS-20	L	M	65	CRC	A	c.203T>G; p.I68S	3	77% (2618/3406)	LOH	LOH	2	WT	Pathogenic mutation and LOH
						c.350C>T; p.T117M	5	38% (147/382)	ROH				
sLS-21	L	M	71	CRC	A	c.194G>A; p.G65D	4	64% (3064/4823)	LOH	LOH (1 marker)	2	WT	Likely pathogenic mutation and LOH
sLS-22	L	M	52	CRC	A	c.2042C>T; p.A681V	3	81% (438/538)	LOH	LOH	2	WT	VUS and LOH
sLS-23	L	M	58	CRC	A	*MSH6*: c.412C>A; p.P138T	3	32% (664/2066)	ROH	ROH (1 marker, *MSH6*), ROH (*MLH1*)	2 (*MLH1* & *MSH6*)	c.1799T>A; p.V600E	1 VUS
sLS-24	L	M	27	CRC	A	c.1600-1601delinsAG; p.V534R	3	38% (1410/3727)	ROH	ROH	2	WT	1 VUS
MSH2													
sLS-25	C	F	34	Ovary	B	GL: c.1147C>T; p.R383*	5	86% (7777/9064)	LOH	LOH (1 marker)	2	WT	Germline mutation
						c.965G>A; p.G322D (only PGM)	1	21% (801/3832)	ND				
sLS-26	C	F	39	EC	B	GL: c.1147C>T; p.R383*	5	39% (1126/2868)	ROH	Partial LOH	2	WT	Germline mutation
sLS-27	C	M	61	CRC	A	c.818delT; p.V273fs	5	82% (1644/2000)	LOH	NI	2	NE	Pathogenic mutation and LOH
sLS-28	C	F	36	CRC	A	no variant detected				Partial LOH	2	WT	LOH
sLS-29	C	M	64	CRC	A	no variant detected				LOH	2	WT	LOH
sLS-30	C	F	38	CRC	A	no variant detected				LOH	2	WT	LOH
sLS-31	C	F	47	CRC	A	no variant detected				ROH	2	WT	No MMR aberrations
sLS-32	L	M	66	CRC	A	c.83insG; p.E28fs	5	40% (158/398)	ROH	ROH	2	WT	2 pathogenic mutations
						c.514G>A; p.K172*	5	49% (1091/2209)	ROH				
sLS-33	L	F	53	EC	A	c.255delT; p.N85fs*2	5	42% (842/1996)	NE	ROH	2	WT	2 pathogenic mutations
						c.2145dupT; p.D716*	5	37% (571/1559)	ROH				
sLS-34	L	F	58	EC	A	c.1903A>T; p.K635*	5	88% (3650/4150)	LOH	LOH	2	WT	Pathogenic mutation and LOH
sLS-35	L	F	49	CRC	A	c.1601delA; p.N538fs	5	84% (1670/2000)	LOH	NI	ND	WT	Pathogenic mutation and LOH
sLS-36	L	M	55	CRC	A	c.279_281delTCT; p.L94del	1	40% (791/2000)	NE	LOH	2	WT	VUS and LOH
						c.857T>C; p.F286S	3	39% (1186/3078)	ROH				

Table 1. Continued.

Patient	Cohort	M/F	Age[a]	Tumor	Germline analysis	Somatic variants	Class (1-5)	% of variant reads with PGM (variant reads/ total reads)	LOH/ROH Sanger	LOH/ROH SNaPshot	Copy number FISH	*BRAF*	Conclusion somatic MMR aberrations
MSH6													
sLS-37	C	M	79	CRC	B	c.2672_2673delTC; p.I891fs	5	41% (615/1498)	ROH	ROH	2	WT	VUS and pathogenic mutation
						c.3725G>A; p.R1242H	3	41% (3204/7884)	ROH				
sLS-38	C	M	37	CRC	A	no evaluable results		PGM results NE		ROH	NE	NE	No MMR aberrations
PMS2													
sLS-39	L	F	69	EC	A	c.943C>T; p.R315*	5	34% (923/2732)	NE	ROH (low tumor%)	2	WT	1 pathogenic mutation
						MLH1: c.1469T>C; p.M490T	2	26% (495/1905)	ROH				
						MSH2: c.1448A>G; p.E483G	2	43% (211/486)	ROH				
						MSH6: c.1054G>A; p.V352I	2	34% (67/200)	ROH				
no deficient MMR protein													
sLS-40	L	M	53	CRC	A	*MSH6*: c.3724C>T; p.R1242C	3	34% (1660/4902)	ROH	ROH (*MSH6*)	2 (all genes)	NE	1 VUS

Patients were categorized according to the affected mismatch repair (MMR) gene, as indicated by immunohistochemistry; all somatic aberrations (except for *BRAF*) were detected in the corresponding MMR gene unless specified otherwise. For germline testing, patients were classified as: (A) index patient tested for germline MMR gene mutations; or (B) index patient deceased, first degree relative(s) tested for germline MMR gene mutations. Somatic variants were classified as: benign (1), likely not pathogenic (2), uncertain (3), likely pathogenic (4) or definitely pathogenic (5)[19,20].

[a]At time of diagnosis

C: clinical genetics diagnostic cohort; CRC: colorectal cancer; EC: endometrial cancer; F: female; FISH: fluorescence in situ hybridization; L: LIMO study cohort; LOH: loss of heterozygosity; M: male; MMR: mismatch repair; ND: non-determined; NE: non-evaluable; NI: non-informative; ROH: retention of heterozygosity; sLS: suspected Lynch syndrome; VUS: variant of unknown significance; WT: wild-type

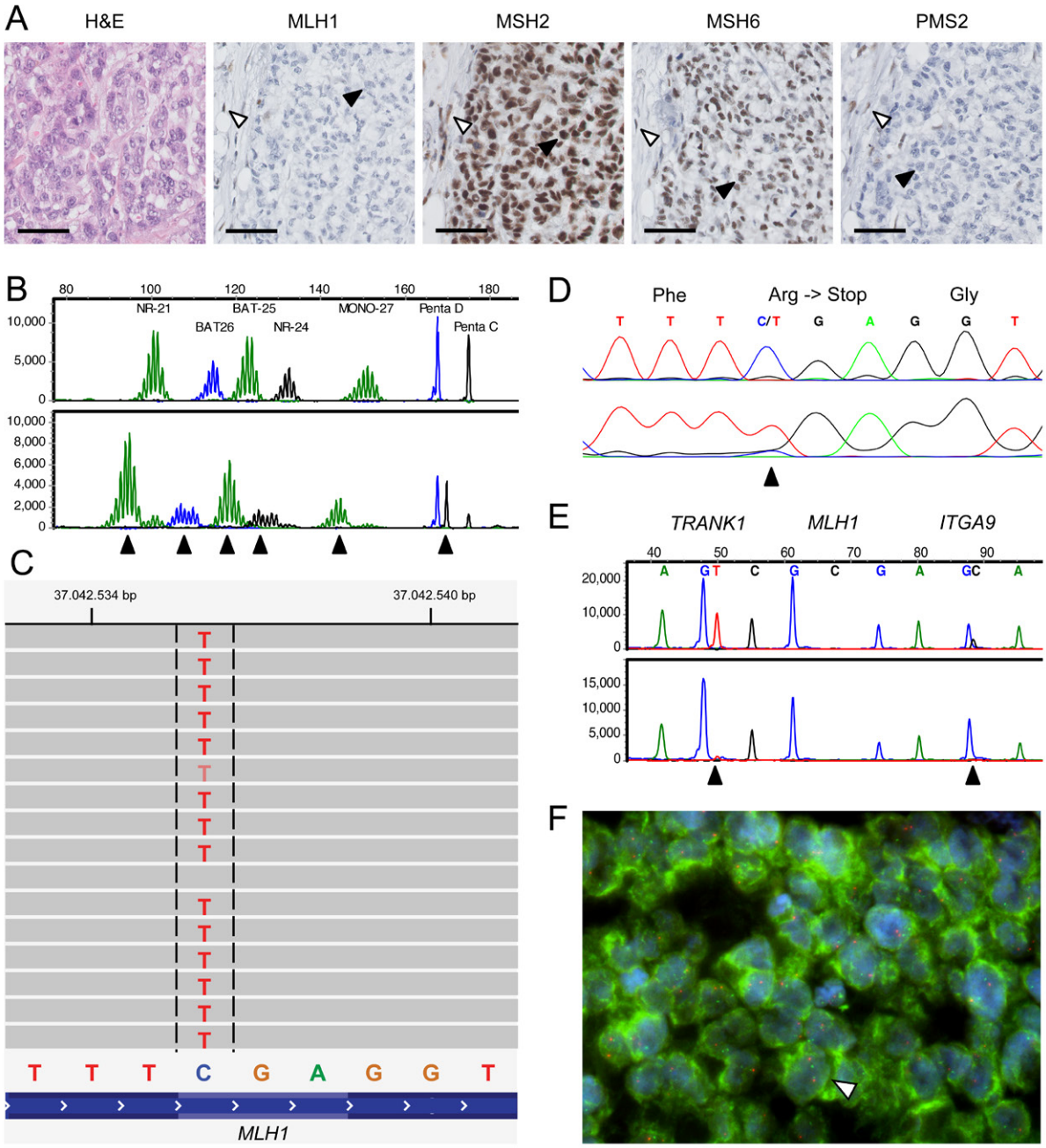

Figure 4. Somatic aberrations for patient sLS-4, who was diagnosed with a moderately–poorly differentiated adenocarcinoma of the colon. (a) Tumor cells show absence of MLH1 and PMS2 expression and normal MSH2 and MSH6 expression (filled arrowheads), stromal cells show expression of all four proteins (open arrowheads); scale bar=50 µm. (b) Microsatellite instability (MSI) analysis shows MSI of six markers (NR-21, BAT-26, BAT-25, NR-24, MONO-27 and Penta C) in the tumor (lower panel) compared to normal (upper panel); the MSI shifts are indicated by arrowheads. (c) A nonsense mutation (c.298C>T) in *MLH1* was detected with the ion torrent personal genome machine. (d) Sanger sequencing confirmed the presence of the mutation (arrowhead) in tumor tissue (lower panel) and shows the absence of the mutation in normal tissue (upper panel). At the location of the mutation, loss of the wild-type allele was detected. (e) LOH was confirmed by SNaPshot analysis; one marker in *TRANK1* and one marker in *ITGA9* (arrowheads) are heterozygous in normal tissue (upper panel) and show LOH in tumor tissue (lower panel). (f) Copy number analysis by fluorescence in situ hybridization shows two copies of the *MLH1* locus (red signal) and two copies of a control locus on the opposite arm of chromosome 3 (green signal) in the tumor cells (open arrowhead).

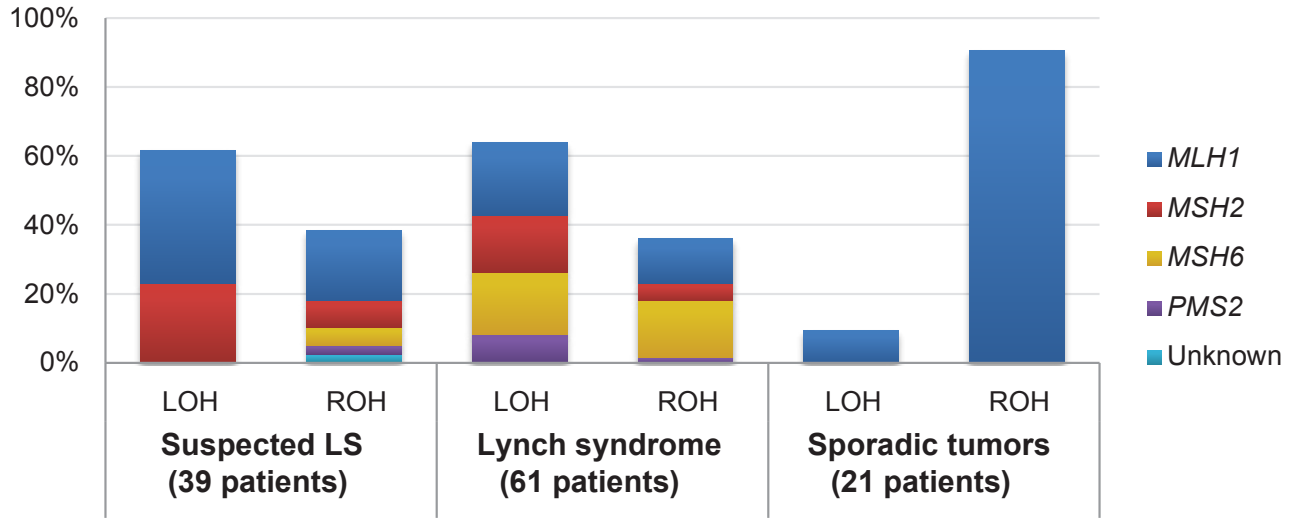

Figure 5. Percentages of tumors with LOH or ROH for suspected Lynch syndrome (sLS) patients, Lynch syndrome patients and patients with sporadic tumors; different colors indicate the affected mismatch repair genes (for sLS patients, as indicated by immunohistochemistry).

LOH: loss of heterozygosity; ROH: retention of heterozygosity

showed LOH of (part of) the affected gene (Figure 5). For the tumors of sLS patients, LOH was detected in 15/23 (65%) and 9/12 (75%) of the MLH1- and MSH2-deficient tumors, respectively. For 36 variants detected in tumors of sLS patients, both PGM data and LOH results by Sanger sequencing were available; for these variants, the percentage of variant reads by PGM was compared to LOH results by Sanger sequencing (Table 1); 15/36 variants showed LOH by both PGM and Sanger sequencing, 20/36 showed ROH by both PGM and Sanger sequencing, and one showed LOH by PGM (81% variant reads) but ROH by Sanger sequencing. Interestingly, the tumor of this patient (sLS-8) did show LOH by SNaPshot analysis. For the LS-associated tumors, LOH was detected in 13/21 (62%), 10/13 (77%), 11/21 (52%) and 5/6 (83%) of the tumors of *MLH1*, *MSH2*, *MSH6* and *PMS2* germline mutation carriers, respectively. Overall, seven tumors showed partial LOH by SNaPshot analysis and another eight tumors showed LOH by SNaPshot analysis but ROH by Sanger sequencing, with at least one variant.

Copy number analysis

All tumors were screened with FISH to detect MMR gene copy number variations: 121 tumors showed two copies of the affected MMR gene; two tumors showed only one copy; one tumor showed polysomy; nine tumors showed non-evaluable results; and for two tumors copy number by FISH could not be determined. Both tumors with only one copy of the affected MMR gene (patients LS-27 and LS-56) showed LOH by SNaPshot analysis.

BRAF mutation analysis

All tumors were screened for *BRAF* mutations, using a sensitive mutation-specific PCR to detect V600E and V600K mutations and Sanger sequencing. For 127 tumors *BRAF* analysis had evaluable results. Combining the results from both assays, *BRAF* mutations were detected in 5/34 (15%) tumors of sLS patients, 1/67 (1%) LS-associated tumors and 22/26 (85%) sporadic tumors. V600E mutations were detected in three tumors of sLS patients and in 22 sporadic tumors, a K601E mutation was detected in the tumor of a sLS patient and a *BRAF* D594G mutation was detected in both a LS-associated tumor and the tumor of a sLS patient.

Discussion

Somatic MMR gene aberrations were investigated in 40 tumors of sLS patients negative for germline mutations in the affected MMR gene(s), as indicated by IHC; final conclusions are shown in Figure 6. Two

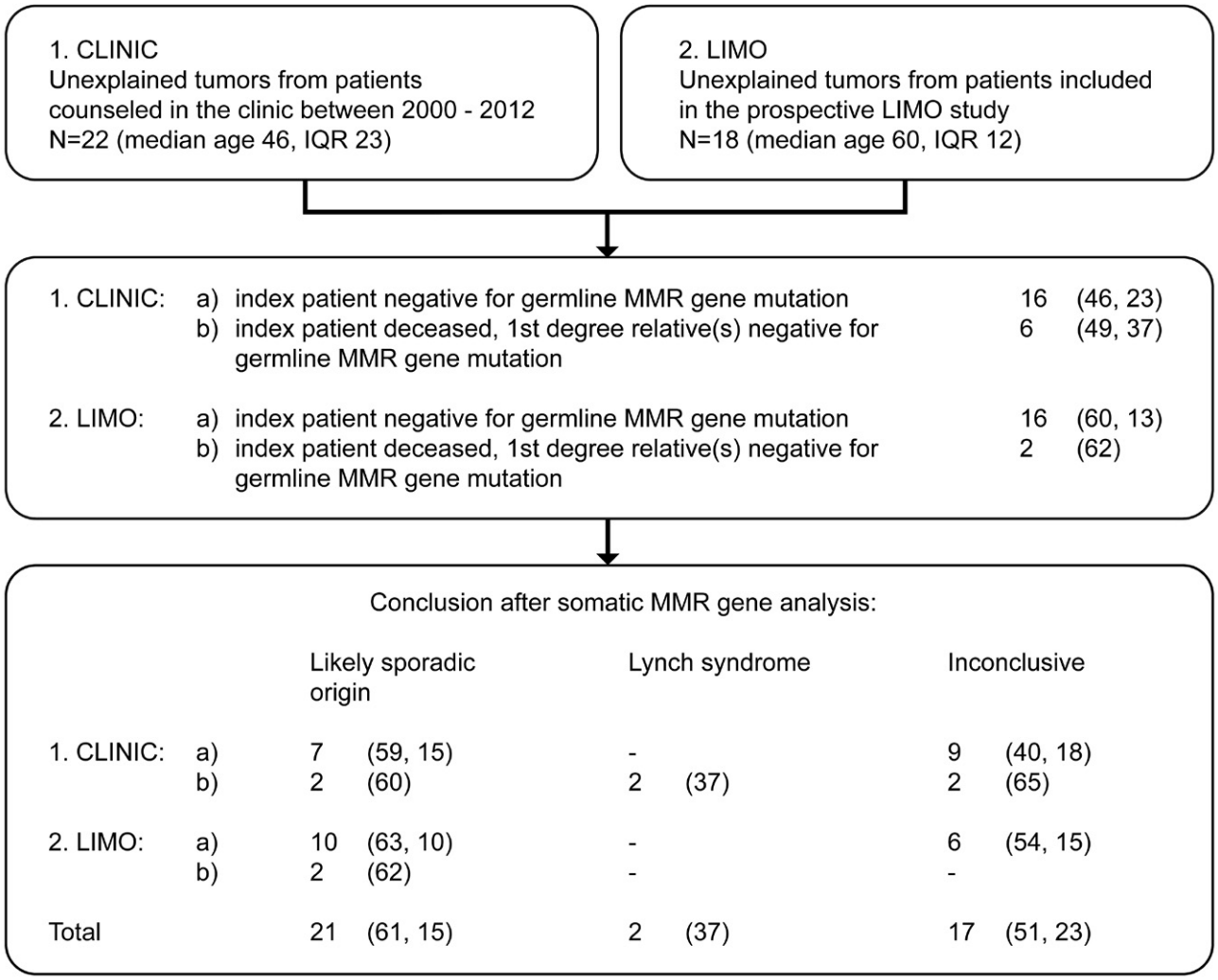

Figure 6. Suspected Lynch syndrome patient details and final conclusions based on tumor analysis; numbers of patients (median age, IQR) are shown for all patient groups.

MMR: mismatch repair; n: number of patients

somatic and (likely) deleterious aberrations of the affected MMR genes were detected in 21/40 (53%) of these tumors, 16/24 for *MLH1* and 5/12 for *MSH2* (Figure 4). In addition, 5/40 (13%) patients showed a variant of unknown pathogenicity combined with a pathogenic mutation or LOH. No pathogenic mutations were detected in the non-affected MMR genes, as indicated by IHC. This suggests that secondary mutations in the non-affected MMR genes are uncommon. Furthermore, 19/21 (91%) tumors with *MLH1* promoter hypermethylation showed ROH for the *MLH1* gene, which is in accordance with the notions that microsatellite-unstable tumors are generally chromosomally-stable[21] and that *MLH1* promoter hypermethylation affects both alleles[22].

Focusing on the MLH1- and MSH2-deficient tumors, 21/36 (58%) tumors had two somatic and (likely) deleterious aberrations. This is comparable to the study of Mensenkamp et al.[12], who identified two somatic aberrations in 13/25 (52%) MLH1- or MSH2-deficient tumors. In the current study, 5/36 (14%) of the tumors showed two pathogenic mutations and 16/36 (44%) showed a combination of a (likely) pathogenic mutation and LOH. For the study of Mensenkamp et al.[12], this was 5/25 (20%) and 8/25 (32%) respectively; the slightly lower proportion of tumors with a pathogenic mutation and LOH could be explained by the fact that for 10/25 tumors, LOH

analysis was not informative. We did not observe a different percentage of likely sporadic tumors for CRCs (55%, 18/33) compared to ECs (60%, 3/5). Mensenkamp et al.[12] showed that 48% (11/23) of CRCs and 100% (2/2) of ECs were likely of sporadic origin. Although both studies showed that EC can be caused by two somatic aberrations, the numbers of ECs included are too low to reliably compare the distribution of somatic aberrations between CRCs and ECs.

For two related sLS patients (sLS-25 and sLS-26, sisters) the same pathogenic *MSH2* mutation was found in both normal and tumor tissues, indicating a germline predisposition. From these patients no blood DNA was available as a source of constitutional DNA, since both patients were deceased at time of germline mutation analysis. Four of their healthy children were tested and no germline mutations in *MLH1*, *MSH2* or *MSH6* were found, indicating that these children did not inherit the *MSH2* germline mutation from their mothers. This exemplifies that mutation analysis of normal and tumor DNA isolated from archival FFPE tissue can be a valuable approach for LS testing in patients from whom no blood DNA is available.

In the tumors of 21 sLS patients, two (likely) deleterious somatic aberrations were detected, either two mutations or one mutation and LOH. It is likely that these aberrations are located on different alleles, causing bi-allelic inactivation of the MMR gene involved. For 12/16 tumors with a mutation and LOH, loss of the wild-type allele could indeed be confirmed by Sanger sequencing. These tumors may now be considered not to be associated to Lynch syndrome. As these patients are no longer suspect for LS, extensive colonoscopic surveillance similar to that needed in LS patients is no longer required. The starting age and frequency of colonoscopies for these patients and their relatives can now be solely based on family history.

Some of the sLS patients included in the current study were previously involved in the prospective multicenter LIMO study (Figure 1), in which all consecutive CRC and EC patients≤70 years were screened for LS[7,8]. In total, 1117 CRCs and 179 ECs were screened and germline mutation analysis was performed for 52 suspected LS patients: 34 (65%) patients had a germline MMR mutation or VUS and for 18 (35%) patients no mutations were detected. We screened the tumors of these 18 patients without germline MMR mutations for somatic aberrations of the MMR genes: 12 tumors (10 CRCs and two ECs) had a likely sporadic origin, and for six tumors (five CRCs and one EC) the results were inconclusive (Figure 6). Thus, 12/52 (23%) patients who were referred to the Clinical Genetics Department and tested for germline MMR gene mutations actually had (likely) sporadic tumors.

Only two tumors showed the absence of one of the MMR alleles by copy number analysis, whereas LOH was found in 65 tumors. This suggests that the LOH detected is due to copy-neutral LOH (cnLOH). Previous studies have reported that cnLOH is an important mutational event in the carcinogenesis of microsatellite-unstable tumors and usually confined to the locus harboring pathogenic MMR gene mutations[21,23]. Interestingly, cnLOH was less frequently observed in tumors of *MSH6* mutation carriers[21,23], which corresponds to our findings in LS patients, where LOH is observed in only 11/21 (52%) of tumors of *MSH6* mutation carriers, but in 13/21 (62%), 10/13 (77%) and 5/6 (83%) of tumors of *MLH1*, *MSH2* and *PMS2* mutation carriers, respectively. This suggests that the second hit in *MSH6*-affected tumors is less often loss of the wild-type allele, but may be a second somatic mutation. An alternative explanation for the absence of copy number alterations is that only a small part of the chromosome is lost, which is not detected by our FISH probes. In 15/65 (23%) tumors we indeed found indications for partial LOH of the involved MMR gene.

BRAF mutation status is regularly used to distinguish LS-associated tumors from sporadic microsatellite unstable colon cancer, as *BRAF* mutations are correlated with *MLH1* methylation and are strong predictors of MMR gene mutation-negative status[24,25]. In none of the tumors from LS patients was a *BRAF* V600E mutation detected; however, one germline *MSH6* mutation carrier showed a *BRAF* D594G mutation in the tumor. The same mutation was detected in the tumor of a sLS patient. This mutation appears to be a low-activity

mutant[26] and has been described previously in CRC[27,28], but the significance of this mutant in the screening for LS is unknown. In total, *BRAF* mutation status was determined in 15 likely sporadic MLH1-deficient tumors of sLS patients; interestingly, 3/15 (20%) showed a *BRAF* mutation (V600E, K601E and D594G). As these tumors showed no *MLH1* promoter hypermethylation, *BRAF* screening could be valuable in this subgroup of patients to predict the sporadic origin of the tumors.

In 12/40 (30%) tumors of sLS patients, no or only one somatic mutation was found in the tumor. Obviously, some aberrations escaped detection by our analyses, so no final diagnosis with regard to LS could be made for these cases. Other mechanisms, such as mutations in untranslated or (deep) intronic regions, large deletions or alterations in other genes that are involved in regulation or expression of the MMR genes, might be involved in these tumors. Recently, the risk of cancer in families of sLS patients without germline mutations was determined by Rodriguez-Soler et al.[29], who found that the risk of CRC is lower in families with sLS than among patients with genetically confirmed LS, but significantly higher than in cases of truly sporadic CRC. Therefore, sLS patients should only be released from cancer surveillance programs when two somatic hits are detected in the tumor, as any undetected hit could be a germline mutation.

The current study also has some limitations. Some somatic aberrations might have escaped our detection methods; therefore, the number of somatic aberrations of the MMR genes could be underestimated. For the LOH analyses, 12/135 patients showed non-evaluable results, probably due to the use of DNA extracted from FFPE tissue. Furthermore, 9/135 patients were homozygous for all investigated SNPs. As LOH might be confined to only a small region of the MMR gene, it could have been missed due to insufficient informative markers. Additionally, some somatic mutations might have been missed due to the design of the PGM primer panel, as not all exonic regions were completely covered. We did not have a sufficient amount of DNA to analyze all tumors of sLS patients for MMR mutations using an alternative method, therefore we do not know the false-negativity rate for the PGM analysis. The tumors of three sLS patients (sLS-2, sLS-13 and sLS-38) had non-evaluable PGM results (<80% of the target bases were covered>100 times). For two of those tumors (sLS-13 and sLS-38), no conventional Sanger sequencing could be performed as an alternative, due to a limited amount of DNA. Despite the low coverage, the tumor of patient sLS-13 did show one likely benign variant, but Sanger sequencing of this region could not be performed due to low-quality DNA. As the PGM coverage for these patients is very low, potential mutations could have been missed.

In 26/40 (65%) tumors of sLS patients, two or more somatic MMR gene aberrations were found. For 21 patients this concerned (likely) pathogenic mutations, indicating the sporadic origin of the tumors. This result indicates that LOH and somatic mutation analyses of the MMR genes in tumors of sLS patients adds substantially to the final diagnostics of these patients and their relatives. Therefore, we propose to add somatic molecular analyses of the MMR genes to the routine molecular diagnostic workflow of tumors of sLS patients. To better document the incidence of somatic MMR mutations, intronic regions and regions that were not covered in the current design should be analyzed as well. Implementation of whole-genome sequencing might help to identify unknown germline or somatic aberrations associated with LS.

Acknowledgements

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Supplementary Methods

Microsatellite instability (MSI) analysis, mismatch repair (MMR) protein immunohistochemistry (IHC) and *MLH1*, *MSH2*, and *MSH6* promoter hypermethylation assay

MSI analysis was performed on all tumor samples using a panel of five mononucleotide microsatellite markers (Promega pentaplex assay, Promega, Madison, WI). Tumors with more than one unstable

marker were categorized as having MSI. IHC for the MMR proteins was performed as previously described[6]. For suspected Lynch syndrome (sLS) patients, the methylation status of the *MLH1*, *MSH2*, or *MSH6* promoter was determined if the tumor had absent MLH1, MSH2, or MSH6 protein expression, respectively. Promoter hypermethylation was determined by the methylation-specific multiplex ligation-dependent probe amplification assay SALSA MS-MLPA Kit ME011-100R for MMR genes (MRC-Holland, Amsterdam, The Netherlands), according to standard protocols.

LOH analysis

Single PCR - and multiplex PCR assays were designed to detect six to nine SNPs in or adjacent to each of the MMR genes. The following SNPs were included in these assays: rs4476463, rs11712098, rs4441609 (*TRANK1*), rs3774341, rs4234259, rs9876116 (*MLH1*), rs199279, rs11709385, rs2434132 (*ITGA9*); rs3815865, rs10209586, rs2347794, rs7607076, rs6757035, rs3732183, rs3764960, rs2059520, rs11684737 (*MSH2*); rs3136228, rs3136245, rs3136265, rs3136282, rs2348244, rs3136329, rs2020911, rs3136354, rs3136359 (*MSH6*); rs62455883 (*RNF216*), rs62456178, rs12702462, rs2286681, rs2286680, and rs12112229 (*PMS2*). Labelled fragments were detected on an ABI 3730xl genetic analyzer (Applied Biosystems, Foster City, CA). Data was analyzed with Genemarker v2.4.0 software (SoftGenetics, State college, PA).

Mutation analysis of the MMR genes and *BRAF*

Mutation analysis with the Ion Torrent Personal Genome machine (PGM) was performed with suppliers materials and protocols (Life Technologies, Carlsbad, CA). DNA concentrations were measured with the Qubit 2.0 Fluorometer and 2.5 to 10 ng DNA input was used. Libraries were made using the Ion AmpliSeq Library Kit 2.0-384 LV according to the Ion Ampliseq Library Preparation User Guide. Template was prepared using the Ion OneTouch 200 Template Kit v2 DL and sequencing was performed with the Ion PGM Sequencing 200 Kit v2 on an Ion 316, 316 v2, or 318 chip. Data was analysed with Variant Caller v3.6 (Life Technologies). Using ANNOVAR[30] in a local Galaxy pipeline[31-33], variants were annotated with RefSeq and additional information about the variant was obtained using dbsnp137NonFlagged, COSMIC64, ESP6500_ALL, and 1000g2012apr_ALL.
All Sanger sequence analyses were performed with M13 tailed custom made primers, as previously described[18]. Data was analyzed with Mutation Surveyor v4.0 software (SoftGenetics). For the *BRAF* mutation specific PCR labelled fragments were detected as described for the SNaPshot analysis.
Sequences of all primers and probes are available on request.

Predicting pathogenicity for somatic MMR variants

For all missense variants and in-frame deletions the Consensus InSIGHT classification was searched[19,20]. Furthermore, Align-GVGD[34], SIFT[35], Mutation Taster[36] and Grantham scores[37] were calculated using Alamut software v2.3 (Interactive Biosoftware, Rouen, France). Additionally, all variants were analysed by PolyPhen-2[38], FATHMM[39], CHASM[40], and PROVEAN[41]. Variants with a consensus InSIGHT classification were classified accordingly. For variants not classified by InSIGHT, a literature search was performed and classification was based on in silico tool predictions and functional data if available

Supplementary material on the internet

Supplementary Table 1**.** All results for suspected LS patients (sLS), Lynch syndrome (LS) patients and patients with sporadic tumors.
http://www.niekgeurts.nl/proefschrift/Chapter 9 - Supplementary Table 1.xls

Supplementary Table 2**.** Prediction of pathogenicity for missense variants and in-frame deletions of MLH1, MSH2, and MSH6.
http://www.niekgeurts.nl/proefschrift/Chapter 9 - Supplementary Table 2.xls

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Chapter 10

Summary
Discussion

Summary

The studies in the first part of this thesis focus on tumor clonality determinations. Unique DNA markers or somatic DNA aberrations can be used to reliably define tumor origin or the clonal relationships between multiple tumors in the same patient (multiple primary tumors or metastasized disease). These DNA analyses help with the differential diagnosis and facilitate selecting the appropriate treatment for the patient. Studies in the second part address molecular analyses in the context of hereditary testing. As certain hereditary syndromes are characterized by specific somatic aberrations, screening tumors for these aberrations helps to decide whether or not the patient should be referred for genetic counseling. Furthermore, determining the sporadic origin of a tumor in a patient suspected of a hereditary syndrome can facilitate proper diagnosis for the patient and its relatives. The main findings are summarized below.

Part I - Clonality determinations

In **chapter 2**, a patient with a colorectal carcinoma metastasis in a transplanted liver, detected 18 months after transplantation, is described. Although donor-related tumors are very rare in transplantation patients[1-4], the diagnostic question was raised whether this tumor could originate from the donor, as a colonoscopy was negative for the index patient. DNA short tandem repeat genotyping of tumor tissue indicated unequivocally that the tumor cells were of donor origin. These results show that considering the possibility of a donor-related tumor in patients who received a tissue transplant is crucial for proper therapeutic decision-making.

In **chapter 3**, immunoglobulin heavy (IGH) and K light chain (IGK) gene rearrangements were studied in patients with successive B-cell lymphomas to determine whether these were recurrences or unrelated primary lymphomas. After initial treatment, 25 to 85% of patients with malignant lymphoma relapse following a period of complete remission[5-8]. A second occurrence of lymphoma is usually considered a recurrence, however, it may also be an unrelated primary lymphoma, with different treatment options. IGH and IGK rearrangements were studied for 36 patients with multiple lymphomas, diagnosed within a 5- to 15-year time interval. Lymphoma relapses were mostly recurrences of the primary tumor (89-94% of cases). Therefore, routine investigation of the possible clonal relationship between two successive lymphoma is not warranted. For specific subtypes of lymphoma molecular analysis might be valuable, for example for diffuse large B-cell lymphoma (DLBCL), as the only two successive lymphomas that were unrelated primary lymphomas concerned DLBCL.

In **chapter 4**, the potential use of the mitochondrial DNA (mtDNA) D310 marker for clonality determinations of clinically challenging synchronous or metachronous tumors was examined for a wide range of tumor types. Sanger sequencing of the D310 mononucleotide repeat was performed on a diagnostic cohort of 382 patients with 857 tumors that were previously analyzed using routine molecular analysis on genomic DNA. For 26% of patients a D310 mutation was detected in at least one of their tumors, for these patients the D310 can be used to determine the clonal relationship between their multiple tumors. However, clonality assessments based on mtDNA and genomic DNA were only concordant in 71% of patients. Although D310 mutation status might aid in clonality determinations, as a single assay it has limited predictive value. To further evaluate the potential contribution of mtDNA markers to the assessment of tumor clonality, next generation sequencing (NGS) assays can be complemented with mtDNA markers, such as the D310 repeat.

In **chapter 5**, the accuracy and additional value of targeted NGS for determining the clonal relationship between two lung lesions from the same patient was examined. Histological and molecular subtyping of non-small cell lung cancer (NSCLC) is important for predicting survival and drug response, however, up to 8% of NSCLC is multifocal[9-12]. The two tumors of this patient showed different activating *EGFR* mutations, *EGFR* amplification status, *TP53* mutation status and loss of heterozygosity patterns by routine analysis. Targeted NGS was performed using the commercially available AmpliSeq Cancer Panel, which targets hotspot regions of 50 genes frequently

mutated in multiple tumor types. With NGS, all conventional detected mutations were confirmed, and an additional variant, in a gene not covered by routine analysis, was detected. NGS accurately determined the multifocal NSCLC of this patient as two unrelated primary tumors. Additionally, these results suggest that multifocal NSCLC should be considered as potentially multiple primary tumors and stratification for targeted therapy based on molecular markers should be performed on all tumor foci present.

In **chapter 6,** the value of targeted NGS in the diagnostic workup of *BRCA1/2* gene mutation carriers with more than one tumor location was evaluated. Female *BRCA1/2* gene mutations carriers have a high cumulative lifetime risk for developing breast and ovarian cancer (55-85% and 10-60%, respectively)[13-16] and therefore are often diagnosed with multiple tumors. It is of clinical importance to determine the clonal relationships between these tumors, as the primary tumor guides treatment and determines prognosis[17,18]. For this study, conventional histopathological revision was performed on the multiple tumors of 42 patients, resulting in a conclusive result on tumor origins for 38 patients. For 14 patients targeted NGS was performed, using a custom made primer panel for the detection of mutations as well as DNA copy number changes. For all 14 patients, NGS could unequivocally determine the clonal relationships between the multiple tumors. For 10 of these patients conventional histopathology also yielded a conclusive result, which matched the molecular outcomes in all cases.

Part II - Molecular diagnostics in the context of hereditary testing

In **chapter 7**, β-catenin immunohistochemistry (IHC) and *CTNNB1* mutations were analyzed in 18 pediatric desmoid tumors to identify possible *APC* germline mutation carriers. In 11 tumors, abnormal nuclear β-catenin accumulation was detected, indicative for an *APC* or *CTNNB1* mutation. 7/11 tumors showed somatic *CTNNB1* mutations. In two tumors with abnormal β-catenin staining and no *CTNNB1* mutation, an *APC* mutation was detected, which appeared to be germline. This illustrates that β-catenin IHC and *CTNNB1* mutation analysis are useful tools in selecting pediatric patients with desmoid tumors for germline testing of *APC*.

In **chapter 8**, a pitfall in the current screening strategy for mismatch repair deficiency (MMR-D) is presented. A family was identified in which both the mother and the father were germline *PMS2* mutation carriers, resulting in a 25% chance for their children to be biallelic germline *PMS2* mutation carriers. Biallelic germline MMR gene mutations are associated with constitutional MMR-D (CMMR-D)[19]. Diagnostic strategies for selection of patients for genetic counseling for CMMR-D or Lynch syndrome (LS), include microsatellite instability (MSI) testing and/or MMR protein IHC in tumor tissue[20]. The index patient and his sister had biallelic *PMS2* mutations, which were confirmed to be derived from the father and mother. The brain tumors of both the index patient and his sister showed absence of PMS2 expression. However, MSI analysis showed MSI only for 2/5 markers in the tumor of the index patient, and no MSI in the tumor of the sister. These data show that in case of a clinical suspicion of CMMR-D, MSI analysis is suboptimal and should be routinely combined with MMR protein IHC.

In **chapter 9**, somatic aberrations of the MMR genes were analyzed in microsatellite-unstable tumors of 40 suspected LS patients tested negative for germline MMR gene mutations. Approximately 35% of suspected LS patients test negative for germline MMR gene mutations, hampering conclusive LS diagnosis[21,22]. Tumors were analyzed for somatic MMR gene mutations and for loss of heterozygosity using a custom made targeted NGS panel and a custom made SNaPshot assay. Half of the suspected LS patients negative for germline MMR gene mutations appeared to have sporadic tumors due to two somatic MMR gene aberrations. As a result these patients are no longer suspected of LS and therefore analysis of somatic MMR gene aberrations adds substantially to the final diagnosis of these patients and their relatives. Based on our results and literature[23,24], we advise to add somatic molecular analysis of the MMR genes to the routine molecular workflow of suspected LS patients.

Discussion

Next generation sequencing: possibilities and challenges

The introduction of targeted NGS into the molecular pathology laboratory has led to many changes, and multiple chapters in this thesis are based on NGS technology. One of the most important features of NGS for molecular pathology is that it enables the simultaneous testing of multiple DNA fragments using a limited amount of DNA input (only 10 ng for the Ion Torrent PGM). With conventional Sanger sequencing between 1 and 10 DNA fragments were tested for each tumor, depending on the tumor type. For example, mutation analysis of *BRAF* was performed for melanoma, *KRAS* and *EGFR* for non-small cell lung carcinoma, and *KIT* and *PDGFRA* for gastrointestinal stromal tumors. With NGS mutation analysis, most molecular pathology laboratories in the Netherlands use one general diagnostic panel, including all clinically relevant mutations for multiple tumor types. Especially in the academic hospitals NGS panels are often augmented with additional genomic fragments (genes), which might become diagnostically relevant in the (near) future. Using large NGS panels in diagnostics sometimes results in unexpected findings and/or detection of genomic variants of unknown significance. Appropriate evaluation of these findings with bio-informaticians, pathologists and clinicians is a major challenge.

An advantage of NGS is the high sensitivity compared to Sanger sequencing, which results in the detection of variants present in a low percentage of the cells from which DNA has been isolated. For some analyses this is very useful, for example when it is impossible to obtain DNA from a high percentage of neoplastic cells. Furthermore, some mutations associated with resistance to targeted therapies are only detected in low allele frequencies, and might therefore escape detection using Sanger sequencing. This high sensitivity however also creates a new dilemma: what percentage of the tumor cells need to carry a specific mutation for the tumor to be responsive to targeted therapy?

For clonality determinations, NGS obviously has a great advantage over Sanger sequencing. As many genes can be tested in one NGS analysis, the chance to detect mutations in the tumors is largely increased. This is especially true for tumors of unknown primary, as the chance to detect mutations with Sanger sequencing depends on selection of the appropriate genes. Additionally, copy number aberrations can be analyzed simultaneously in the same NGS panel using single nucleotide polymorphisms (SNPs). The NGS panel used for clonality determinations at the department of Pathology of the Erasmus MC was previously described and targets the entire open reading frame of *CDKN2A*, *PTEN* and *TP53*, multiple hotspots sites for 27 different genes and 143 SNPs at 15 different loci for the detection of copy number aberrations. Use of this panel often results in detection of multiple aberrations, mutations as well as copy number aberrations. This increase in the number of detected aberrations per tumor can however also complicate interpretation. Frequently, common aberrations as well as differences between two tumors are detected. Biologically this makes sense; two independent primary tumors can by chance develop the same aberrations. Especially hotspot mutations or identical copy number aberrations are found in many different tumor types. On the other hand, metastatic disease might be heterogeneous and result in additional aberrations in one or more of the tumor localizations. Classification of tumors as clonally related or not depends on critical evaluation of all identified DNA aberrations in the context of the clinical and pathological characteristics.

For somatic testing in the context of hereditary testing, to detect somatic aberrations that suggest a sporadic origin of the tumor, introduction of NGS was essential. Many hereditary syndromes are caused by mutations in tumor suppressor genes, which need a second hit before the gene is inactivated. Tumor suppressor genes can harbor all types of inactivating mutations including missense, nonsense, frameshift and splice site mutations, but also larger aberrations like exon and whole gene deletions. Tumor suppressor genes usually do not have mutation 'hotpots', therefore, testing for aberrations generally involves screening the complete coding sequence, including the intron-exon boundaries. Routinely sequencing

these genes in DNA isolated from formalin fixed paraffin embedded (FFPE) tissue is challenging, but since the introduction of NGS no longer impossible. Now large tumor suppressor genes, like the MMR genes, are routinely tested for somatic mutations facilitating proper diagnosis for patients and their families. A continuing challenge in somatic testing of tumor suppressor genes using NGS is to detect entire exon deletions and other large genomic aberrations. This can be tested with an additional multiplex ligation-dependent probe amplification analysis, but a combined NGS approach would be more efficient and would require less DNA input.

Beyond next generation sequencing

In this thesis different techniques are described, including short tandem repeat genotyping, IGH and IGK rearrangement analysis, Sanger sequencing, MSI analysis, and targeted NGS. This wide range of techniques is illustrative of the current molecular pathology laboratory, where many different types of analyses are performed on a daily basis. Especially in academic hospitals, these analyses are continuously adapted according to the most recent experimental, clinical and technical findings and/or (experimental) treatments available. Technological developments are an important factor in the ability to incorporate all these new analyses into a routine diagnostic setting. Below, some technological advancements potentially relevant for future molecular pathology are discussed.

Whole exome sequencing (WES) / whole genome sequencing (WGS)

With the introduction of NGS into the routine diagnostic setting, possibilities for mutation analysis seem endless. Most molecular pathology laboratories started with relative small targeted NGS panels, mainly composed of clinically relevant genes. However, as the costs for NGS are dropping, the question arises whether molecular pathology laboratories should be performing WES or WGS instead. This would result in detecting all (potentially) relevant aberrations, without having to select the appropriate genes in advance. The advantages of WES/WGS for research purposes are obvious, as unexpected mutations will only be detected using these approaches. For diagnostic purposes WEG/WGS will undoubtedly result in frequent off-target findings that might be difficult to interpret in a clinical setting, however, it is possible to only analyze the data of genes relevant for the specific diagnostic question. The advantage compared to targeted sequencing is obvious, if any other genes become relevant in the future, the data is already there. A challenge for the application of WES/WGS in solid tumors diagnostics might be generating good quality data using DNA isolated from FFPE tissue.

RNA-seq

The current application of NGS in molecular pathology laboratories is mostly DNA sequencing. However, the same platforms can be used for RNA sequencing, which can especially be useful for the detection of gene fusions resulting from genomic translocations. An illustrative example is the Ion Ampliseq RNA fusion lung cancer research panel from Life Technologies (Thermo Fischer Scientific, Waltham, Massachusetts), which targets over 70 fusion transcripts in one assay. This assay detects known fusion partners, as well as indicates potential translocations with unknown fusion partners. With conventional in situ hybridization (ISH, break-apart probes) usually only a break in one of the target genes is detected, which is however generally sufficient for clinical purposes. Additionally, methods are available for NGS-based gene fusion detection without prior knowledge of the fusion partner (FusionPlex, ArcherDX, Boulder, Colorado).

RNA scope

In ISH a labeled nucleic acid (DNA or RNA) complementary probe is used to detect with subcellular resolution specific DNA or RNA sequences in a tissue section. Currently, most routine diagnostic ISH assays are based on detection of DNA aberrations because of the low sensitivity and/or specificity of RNA ISH. RNA scope (Advanced Cell Diagnostics, Hayward, California) is a novel RNA ISH method that visualizes, down to single molecules, specific RNA sequences in individual cells and enables semi-quantitative in situ gene expression analysis. Multiplexing of different target genes with RNA scope is also possible.

Methylation profiling

DNA methylation denotes the conversion of a cytosine to 5-methylcytosine, which typically occurs at CpG sites (a cytosine 5' of a guanine). Many genes have CpG islands associated with the promoter regions, and methylation of these CpG sites can lead to inhibition of transcriptional activity. As particular tumor types are characterized by specific methylation patterns, methylation profiling can be used for tumor classification. This is especially useful for tumors that are difficult to classify using conventional criteria, like for pediatric brain tumors[25].

One of the main limitations of the techniques mentioned above is the requirement of a sufficient amount of tumor tissue. Removing a piece of tumor tissue is invasive and difficult for certain tumor types. Furthermore, a biopsy might suffer from sample bias due to molecular heterogeneity within the tumor. Recently, several less invasive techniques for molecular profiling of a tumor are being established, some of which are discussed below.

Circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA)

Two main approaches for tumor analysis based on blood are the analysis of CTCs and ctDNA. CTCs are tumor cells that have shed from a tumor, circulate in the bloodstream and can give rise to metastases in distant organs. ctDNA is thought to enter the bloodstream following apoptosis or necrosis of tumor cells. Both DNA from CTCs as well as ctDNA can be used for analysis of the genetic profile of the tumor. The main challenge in analyzing CTCs or ctDNA is the high abundance of circulating normal cells and cell free DNA from normal cells (non-tumor cells). However, very sensitive detection methods are available for detecting specific mutations at low allele frequencies, like the Droplet Digital PCR (Bio-Rad, Hercules, California). For example, the *EGFR* T790M resistance mutation can be monitored by analyzing CTCs or ctDNA[26,27].

RNA-seq of platelets

Blood platelets are circulating anucleated cell fragments and involved in the systemic and local response to tumor growth. When platelets are exposed to tumor cells their RNA profile is altered in a specific manner. These so called tumor-educated platelets have diagnostic potential as they harbor unique RNA profiles depending on the tumor type present in the patient[28,29]. A recent study showed that blood platelets provide a valuable platform for detecting and subtyping multiple types of cancer[30].

Molecular pathology in clinical decision-making

Different disciplines are involved in clinical decision-making and for the clinical scientist in molecular pathology the main interactions are with pathologists, clinicians and clinical geneticists. Traditionally, a pathologist authorizes molecular results before being reported to the clinic. In some hospitals however, including the Erasmus MC in Rotterdam, the clinical scientist in molecular pathology reports the results of molecular testing for targeted treatment directly to the requesting clinician, without interference of a pathologist. The main reason for this is the need to report the results as quickly as possible to the clinic in order to avoid any delay in start of the treatment. However, also for these tests, involvement of a pathologist is imperative to indicate the appropriate tissue region for testing and identifying the tumor cells from which DNA should be isolated. For many complex molecular analyses, like tumor clonality determinations and other differential diagnostic questions, the requesting pathologist is in the lead and integrates the molecular results with clinical, histopathological and immunohistochemical findings to make an optimal diagnosis.

In the last few years many clinical scientists in molecular pathology in the Netherlands have become involved in multidisciplinary meetings with pathologists and/or clinicians. In these meetings specific patients with unusual molecular profiles are discussed, not limited to the molecular details, but also including treatment options and outcome. These meetings underscore the importance of molecular testing in patient care, and are mutually instructive for both clinicians and clinical scientists in molecular pathology. As more and more targeted treatment possibilities are developed, close collaboration between pathologists, clinicians and clinical scientists

in molecular pathology is crucial to keep up to date and to deliver the best patient care possible.

In the Netherlands, germline testing is only performed after counseling by clinical geneticists. For certain hereditary syndromes, like Lynch syndrome, tumors are first screened for somatic aberrations to determine whether or not a patient should be referred to clinical genetics. Lately however, the strict separation between somatic and germline testing is fading, which is exemplified by *BRCA* testing in ovarian cancer. Patients with ovarian cancer have a high risk of being a germline *BRCA1* or *BRCA2* mutation carrier[31] and are therefore conventionally counseled and tested for mutations by clinical genetics. Recently however, somatic *BRCA* mutations have become of interest because ovarian tumors with either germline or somatic *BRCA* mutations appear to respond well to PARP inhibitors[32,33]. The most efficient workflow is to first test the tumor for mutations, as both somatic and germline mutations can be detected in tumor DNA, and thus double testing is avoided. Based on these results patients can be stratified for treatment. Subsequently, patients with mutation positive tumors need to be referred to clinical genetics for counseling and germline testing for the specific mutations, as these are potentially germline. In several hospitals in the Netherlands the molecular pathology laboratory is already performing primary testing of ovarian cancer for *BRCA* mutations. As clinical geneticists are very experienced in both testing genes associated with hereditary syndromes as well as interpreting the variants detected, their involvement in implementing somatic testing of these genes is essential.

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Chapter 11

Appendices

Nederlandse samenvatting

List of publications

Curriculum Vitae

PhD Portfolio

Dankwoord

Nederlandse samenvatting

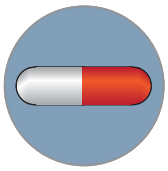
Pathologie is de medische discipline die ziektes diagnosticeert door middel van het onderzoeken van weefsels, cellen en lichaamsvloeistoffen. Deze diagnose is vaak de basis voor de juiste behandeling van de patiënt. Kleine gekleurde weefselfragmenten worden onder de microscoop bekeken om de cellulaire en nucleaire details te beoordelen. Indien nodig worden aanvullend immunohistochemische kleuringen gemaakt, die de aan- of afwezigheid van specifieke cellulaire of extracellulaire componenten kunnen aantonen. Voor de beoordeling van tumoren worden deze onderzoeken steeds vaker aangevuld met moleculair onderzoek. Moleculaire pathologie helpt bij het vaststellen van de juiste diagnose, prognose en/of behandeling van patiënten met kanker door middel van het analyseren van DNA (en eventueel RNA) afwijkingen. DNA is een complex molecuul dat alle informatie bevat benodigd voor de ontwikkeling, het functioneren en de reproductie van levende organismen. Deze informatie wordt gecodeerd door vier verschillende basen: adenine, thymine, guanine en cytosine. Humaan DNA is verdeeld over 23 chromosoom paren die zich in de kern van de cel bevinden, daarnaast is een kleine hoeveelheid DNA aanwezig in de mitochondriën. Humaan DNA bestaat uit ongeveer 3 miljard basen, die grotendeels identiek zijn voor alle individuen. Ongeveer 0,5% van het DNA verschilt tussen individuen. Meestal zijn dit 'neutrale varianten' zonder functionele betekenis, bijvoorbeeld veranderingen van één base (SNPs, single nucleotide repeats) of veranderingen in korte herhalingen van basen (STRs, short tandem repeats).

Kanker ontstaat door DNA mutaties, welke in tegenstelling tot neutrale varianten een pathogeen effect hebben. Tumoren verkrijgen mutaties door een vorm van genomische instabiliteit, bijvoorbeeld chromosomale instabiliteit (CIN) of microsatelliet instabiliteit (MSI). CIN wordt gekenmerkt door grote deleties of toenames van hele chromosomen of delen daarvan, MSI door de aanwezigheid van vele mutaties, voornamelijk in korte herhalingen van basen. Tijdens het ontstaan van een tumor zorgt deze instabiliteit voor de activatie van proto-oncogenen en de inactivatie van tumorsuppressorgenen. De meeste tumoren zijn het resultaat van verkregen (somatische) mutaties, deze mutaties kunnen in elke cel van het lichaam ontstaan. Een minderheid van de tumoren ontstaat in de context van een erfelijk kanker syndroom, waarbij een mutatie geërfd wordt van een van de ouders. Deze geërfde mutaties worden kiembaanmutaties genoemd, en zijn meestal aanwezig in alle cellen van het aangedane individu. De aanwezigheid van deze kiembaanmutatie verhoogt de kans dat het aangedane individu kanker zal ontwikkelen.

Diagnostische moleculaire pathologie

Er zijn verschillende indicaties voor het testen van het DNA van tumoren op de aanwezigheid van somatisch afwijkingen:

Het selecteren van patiënten voor (gerichte) behandeling

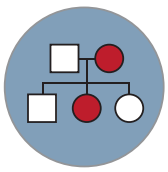


Voor verschillende tumorsoorten zijn gerichte behandelingen mogelijk wanneer specifieke DNA afwijkingen aan- of afwezig zijn.

Differentiaal diagnostisch

Sommige tumorsoorten worden gekenmerkt door de aanwezigheid van specifieke DNA afwijkingen. Wanneer histopathologisch onderzoek niet conclusief is, kan het al dan niet aantonen van deze kenmerkende afwijkingen helpen bij het stellen van de juiste diagnose.

In het kader van erfelijk onderzoek

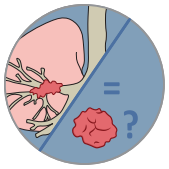


Erfelijke syndromen worden soms gekenmerkt door specifieke somatische afwijkingen. Het aantonen van deze afwijkingen kan ondersteunend zijn in de beslissing om een patiënt door te sturen voor erfelijkheidsonderzoek bij de afdeling klinische genetica.

De moleculaire diagnostiek is de afgelopen jaren een steeds belangrijkere rol gaan spelen in de patiëntenzorg. Sinds 2013 bestaat er dan ook binnen

de Nederlandse Vereniging voor Pathologie (NVVP) een 2-jarige opleiding tot klinisch moleculair bioloog in de pathologie (KMBP). Richtlijnen vastgesteld in 2014 door de NVVP stellen dat elk laboratorium dat moleculaire diagnostiek verricht binnen Nederland een KMBP in dienst moet hebben, of tenminste toegang moet hebben tot KMBP expertise. Dit is belangrijk omdat de moleculaire pathologie een snel veranderend veld is; dagelijks worden nieuwe ontdekkingen en technieken in de routine diagnostiek geïmplementeerd. Een van de meest belangrijke recente veranderingen in de moleculaire pathologie is de introductie van next generation sequencing (NGS). Het principe van NGS is dat, in tegenstelling tot bij Sanger sequentie analyse, miljoenen korte DNA fragmenten tegelijkertijd kunnen worden geanalyseerd. Daardoor is het mogelijk om een grote hoeveelheid genen te analyseren met een hoge sensitiviteit, zelfs wanneer slechts een kleine hoeveelheid weefsel voor analyse beschikbaar is. Aanvullend kan NGS ook gebruikt worden voor het detecteren van DNA copy number afwijkingen (deleties of toenames van DNA), door het analyseren van SNPs of fragment coverage.

Deel I - Clonaliteits-analyses



Sommige patiënten hebben meerdere tumoren, tegelijkertijd of met enige tijd tussen het ontstaan van de tumoren. Deze tumoren kunnen onafhankelijke primaire tumoren zijn, het is echter ook mogelijk dat het een primaire tumor betreft met een uitzaaiing daarvan. Voor optimale behandeling van de patiënt is het belangrijk dit onderscheid te kunnen maken, echter dit is niet altijd mogelijk op basis van de klinische en histopathologische kenmerken. Omdat elke tumor specifieke verkregen (somatische) afwijkingen heeft, kan analyse van deze afwijkingen bijdragen aan het stellen van de juiste diagnose. Wanneer meerdere tumoren dezelfde afwijkingen hebben suggereert dit een gezamenlijke origine, terwijl aanwezigheid van verschillende afwijkingen erop wijst dat de tumoren onafhankelijke entiteiten zijn. Naast somatische afwijkingen kunnen ook andere unieke karakteristieken van de cel geanalyseerd worden. Voor de meeste clonaliteits-analyses binnen de moleculaire pathologie worden tumoren gescreend voor zowel mutaties in multipele genen als voor DNA copy number afwijkingen.

In **hoofdstuk 2** wordt een patiënt beschreven met een uitzaaiing van een colorectaal carcinoom in een getransplanteerde lever. Vanwege een negatieve coloscopie rees de vraag of de tumor van deze patiënt wellicht van donor-origine kon zijn. Unieke patiënt specifieke DNA markers (STRs) zijn geanalyseerd om te bepalen of de uitzaaiing van donor of receptor origine was, de tumorcellen bleken van donor origine te zijn. Deze resultaten laten zien dat ondanks dat donor gerelateerde tumoren erg zeldzaam zijn, het voor de juiste behandeling cruciaal is deze optie te overwegen.

Clonale markers met een hoge voorspellende waarde zijn immuunglobuline zware (IGH) en K lichte keten (IGK) herschikkingen bij lymfomen. Deze DNA herschikkingen zijn niet tumor-specifiek, alle lymfocyten hebben unieke IGH en IGK herschikkingen. Wanneer echter een lymfoom ontstaat uit een van deze lymfocyten, zullen alle lymfoom cellen dezelfde unieke herschikte DNA sequentie bevatten. In **hoofdstuk 3** zijn IGH en IGK herschikkingen bestudeerd in patiënten met meerdere B-cel lymfomen om te bepalen of het multipele primaire lymfomen of een primaire tumor met een of meerdere uitzaaiing(en) betrof. Na initiële behandeling wordt 25-85% van de patiënten met maligne lymfomen opnieuw met een lymfoom gediagnosticeerd, welke meestal beschouwd wordt als dezelfde origine. Voor deze studie zijn 36 patiënten geselecteerd met 5-15 jaar tussen het ontstaan van de opeenvolgende lymfomen, omdat vooral bij een lange periode de kans bestaat dat het meerdere primaire tumoren betreft. In 89-94% van de gevallen bleken de multipele lymfomen echter dezelfde origine te hebben. Dit onderzoek toont daarom aan dat het routinematig onderzoeken van de clonale relatie tussen meerdere opeenvolgende lymfomen niet geïndiceerd is. Dit is mogelijk wel het geval voor specifieke subtypes lymfomen, zoals diffuus grootcellige B-cel lymfomen.

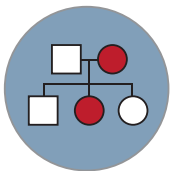
Naast genomisch DNA bevatten humane cellen ook mitochondriaal DNA (mtDNA), waarin voor

meerdere tumortypen afwijkingen zijn beschreven. In **hoofdstuk 4** wordt de mogelijkheid onderzocht om mtDNA afwijkingen te gebruiken voor clonaliteits-analyse bij patiënten met meerdere tumoren. Sanger sequentie analyse van de D310 repeat, een van de meest voorkomende mtDNA afwijkingen, is daarvoor uitgevoerd op een diagnostisch cohort van 382 patiënten met in totaal 857 tumoren, welke eerder zijn onderzocht met de routine moleculaire analyse op genomisch DNA. D310 mutaties werden gedetecteerd in één of meerdere tumoren van 26% van de patiënten, voor deze patiënten kan D310 als clonaliteits-marker worden gebruikt. Echter, de clonaliteits bepalingen op basis van mtDNA en genomisch DNA kwamen slechts tot hetzelfde resultaat in 71% van de patiënten. Deze studie toont aan dat D310 mutatie analyse kan helpen bij clonaliteits-analyses, maar als afzonderlijke test een beperkte voorspellende waarde heeft. Om verder te kijken naar de waarde van mtDNA analyse in clonaliteits-analyses, zouden NGS assays aangevuld kunnen worden met mtDNA markers, zoals de D310 repeat.

In **hoofdstuk 5** wordt de juistheid en de additionele waarde van targeted NGS onderzocht voor het bepalen van de clonale relatie tussen twee long laesies van een patiënt. Het sub-typeren van niet-kleincellige longtumoren door middel van histologie en moleculair onderzoek is belangrijk voor het voorspellen van prognose en therapierespons. De twee tumoren van de index patiënt lieten verschillende activerende *EGFR* mutaties, *EGFR* amplificatie status, *TP53* mutatie status en chromosomale verliezen zien met routine moleculaire analyse. Met NGS, waarmee gescreend wordt op mutaties in de hotspots van 50 genen, werden alle eerder gevonden mutaties bevestigd. Aanvullend werd een extra variant gevonden in een gen wat niet gecoverd werd door de routine analyse. Concluderend werden de tumoren met behulp van NGS analyse correct als onafhankelijke primaire tumoren beschouwd. Deze studie toont tevens aan multifocale niet-kleincellige longtumoren als mogelijk meerdere onafhankelijke primaire tumoren moeten worden beschouwd, en dat moleculaire screening voor therapiekeuze op alle laesies zou moeten worden uitgevoerd.

In **hoofdstuk 6** werd de waarde van targeted NGS voor de diagnostiek van patiënten met *BRCA1* of *BRCA2* kiembaanmutaties en twee of meerdere tumoren geëvalueerd. Vrouwelijke *BRCA1* of *BRCA2* kiembaandragers hebben een hoge kans op het ontwikkelen van borst- en ovariumkanker, en ontwikkelen daarom vaak meerdere tumoren. Het is belangrijk de clonale relatie tussen deze tumoren te bepalen, aangezien de behandeling en prognose bepaald worden aan de hand van de primaire tumor. Voor deze studie is routine histopathologie verricht op de tumoren van 42 patiënten, waarbij voor 38 patiënten een conclusief resultaat verkregen werd. Voor 14 patiënten is vervolgens targeted NGS uitgevoerd, met behulp van een custom made panel voor het detecteren van zowel mutaties als copy number veranderingen. Voor alle 14 patiënten konden de relaties tussen de verschillende tumoren eenduidig bepaald worden met behulp van NGS. Voor 10 van deze patiënten was ook een conclusief resultaat aanwezig op basis van histopathologie, welk overeenkwam met het NGS resultaat voor alle patiënten. Deze studie toont aan dat voor de meeste *BRCA1* en *BRCA2* mutatiedraagsters met meerdere tumoren routine histopathologisch onderzoek voldoende is om de origine van de tumoren te bepalen, en dat NGS analyse een waardevolle aanvulling is wanneer histopathologisch onderzoek niet conclusief is.

Deel II - Moleculaire pathologie in het kader van erfelijk onderzoek



Wanneer een patiënt mogelijk drager is van kiembaanmutatie wordt deze binnen Nederland gecounseld door de klinische genetica. Een verdenking op een kiembaanmutatie kan bijvoorbeeld gebaseerd zijn op een jonge leeftijd bij diagnose, het hebben van meerdere tumoren en/of familie geschiedenis. Het aantonen van specifieke somatische afwijkingen kan onderdeel uitmaken van de voorscreening van tumoren die mogelijk ontstaan zijn in het kader van een erfelijke afwijking. Ook wanneer patiënten al worden gecounseld kan somatische analyse geïndiceerd zijn, bijvoorbeeld om meer informatie te krijgen over de mogelijke

pathogeniciteit van kiembaanvarianten. Tevens kan het aantonen van somatische afwijkingen soms helpen bij het uitsluiten van een erfelijke oorzaak van een tumor.

Familiare adenomateuze polyposis (FAP) is een erfelijke aandoening waarbij een groot aantal poliepen in de dikke darm voorkomen, waaruit darmkanker kan ontstaan. FAP wordt veroorzaakt door kiembaan mutaties in *APC*. Desmoid tumoren (tumoren van de weke delen) kunnen een eerste manifestatie zijn van FAP, echter, het testen voor kiembaan *APC* mutaties wordt niet standaard uitgevoerd bij kinderen met desmoid tumoren. In **hoofdstuk 7** wordt met behulp van β-catenine immunohistochemie (IHC) en *CTNNB1* mutatie analyse gezocht naar mogelijke *APC* kiembaan mutatiedragers bij 18 kinderen met desmoid tumoren. In 11 tumoren werd afwijkende β-catenine aankleuring gevonden, indicatief voor een *APC* of *CTNNB1* mutatie, 7 van deze tumoren hadden somatische *CTNNB1* mutaties. In twee tumoren met afwijkende β-catenine aankleuring maar geen *CTNNB1* mutatie, werd een kiembaan *APC* mutatie gedetecteerd. Dit onderzoek laat zien dat de combinatie van β-catenine IHC en *CTNNB1* mutatie analyse een goede strategie is om patiënten te selecteren voor kiembaan *APC* analyse.

Lynch syndroom (LS) is een erfelijke aandoening waarbij patiënten een hoge kans hebben op het ontwikkelen van meerdere typen tumoren, waaronder colorectale en endometrium tumoren. LS wordt veroorzaakt door kiembaanmutaties in één van de vier mismatch repair (MMR) genen. De voorscreening voor LS bestaat uit analyse van een tumor door middel van immunohistochemisch onderzoek naar expressie van de vier MMR eiwitten en/of DNA microsatelliet instabiliteit (MSI, een kenmerk van LS) analyse. In **hoofdstuk 8** wordt een pitfall van deze screeningsstrategie besproken voor een familie met biallelische *PMS2* mutaties. Deze studie beschrijft een patiënt en zijn zus met beide biallelische *PMS2* mutaties, afkomstig van de vader en moeder. De hersentumoren van beide patiënten toonden afwezigheid van PMS2 expressie, passend bij de afwijkingen. Echter, MSI analyse toonde MSI voor slechts 2/5 markers in de tumor van de index patiënt, en geen MSI in de tumor van de zus. Deze resultaten laten zien dat bij verdenking op een biallelische MMR afwijking, MSI analyse gecombineerd zou moeten worden met MMR eiwit IHC.

Patiënten verdacht voor het hebben van LS worden getest op de aanwezigheid van kiembaanmutaties in de MMR genen. Bij 35% van deze patiënten wordt echter geen kiembaanmutatie gevonden. In **hoofdstuk 9** zijn de microsatelliet instabiele tumoren van 40 patiënten, verdacht voor LS maar zonder kiembaan MMR mutatie, getest voor somatische afwijkingen van de MMR genen met behulp van targeted NGS en SNaPshot analyse. De helft van de patiënten bleek sporadische tumoren te hebben die ontstaan zijn door twee somatische MMR-gen afwijkingen. Op basis van deze bevindingen zijn die patiënten niet langer verdacht voor het hebben van LS. Deze studie laat zien dat somatische MMR-gen analyse substantieel kan bijdragen aan de diagnose van patiënten verdacht voor LS en hun familieleden. Op basis van onze resultaten en literatuur adviseren we daarom om somatische MMR-gen analyse toe te voegen aan de routine workflow van patiënten verdacht voor LS.

List of publications

Geurts-Giele WR*, van Verschuer VM*, van Deurzen CH, van Diest PJ, Pedrosa RM, Collée JM, Koppert LB, Seynaeve C, Dinjens WN. Molecular determination of the clonal relationships between multiple tumors in *BRCA1/2*-associated breast and/or ovarian cancer patients is clinically relevant. *equal contribution. *Mod Pathol.* 2016

Geurts-Giele WR, Gathier GH, Atmodimedjo PN, Dubbink HJ, Dinjens WN. Mitochondrial D310 mutation as clonal marker for solid tumors. *Virchows Arch*. 2015;467:595-602.

Geurts-Giele WR*, Leenen CH*, Dubbink HJ, Meijssen IC, Post E, Sleddens HF, Kuipers EJ, Goverde A, van den Ouweland AM, van Lier MG, Steyerberg EW, van Leerdam ME, Wagner A, Dinjens WN. Somatic aberrations of mismatch repair genes as a cause of microsatellite-unstable cancers. *equal contribution. *J Pathol*. 2014;234:548-59.

Geurts-Giele WR, Wolvers-Tettero IL, Dinjens WN, Lam KH, Langerak AW. Successive B-cell lymphomas mostly reflect recurrences rather than unrelated primary lymphomas. *Am J Clin Pathol*. 2013;140:114-26.

Geurts-Giele WR, Dirkx-van der Velden AW, Bartalits NM, Verhoog LC, Hanselaar WE, Dinjens WN. Molecular diagnostics of a single multifocal non-small cell lung cancer case using targeted next generation sequencing. *Virchows Arch*. 2013;462:249-54.

Kattentidt Mouravieva AA, **Geurts-Giele WR**, de Krijger RR, van Noesel MM, van de Ven CP, van den Ouweland AM, Kromosoeto JN, Dinjens WN, Dubbink HJ, Smits R, Wagner A. Identification of Familial Adenomatous Polyposis carriers among children with desmoid tumours. *Eur J Cancer*. 2012;48:1867-74.

Zelinkova Z, **Geurts-Giele WR**, Verheij J, Metselaar H, Dinjens W, Dubbink HJ, Taimr P. Donor-transmitted metastasis of colorectal carcinoma in a transplanted liver. *Transpl Int*. 2012;25:e10-5.

Leenen CH, **Geurts-Giele WR**, Dubbink HJ, Reddingius R, van den Ouweland AM, Tops CM, van de Klift HM, Kuipers EJ, van Leerdam ME, Dinjens WN, Wagner A. Pitfalls in molecular analysis for mismatch repair deficiency in a family with biallelic *PMS2* germline mutations. *Clin Genet*. 2011;80:558-65.

Not related to this thesis

Huijsmans CJ, **Geurts-Giele WR**, Leeijen C, Hazenberg HL, van Beek J, de Wildt C, van der Linden JC, van den Brule, AJ. HPV prevalence in the dutch cervical cancer screening population (dusc study): HPV testing using automated hc2, cobas and aptima workflows. *Submitted*

Jansen AM, Crobach S, **Geurts-Giele WR**, van den Akker BE, Ventayol Garcia M, Ruano D, Nielsen M, Tops CM, Wijnen JT, Hes FJ, van Wezel T, Dinjens WN, Morreau H. Distinct patterns of somatic *APC* mosaicism in unexplained adenomatous polyposis patients. *Gastroenterology*, 2016

Rigter LS, Snaebjornsson P, Rosenberg EH, Atmodimedjo PN, Aleman BM, ten Hoeve J, **Geurts-Giele WR**, PALGA group, van Ravesteyn TW, Hoeksel J, Meijer GA, te Riele H, van Leeuwen FE, Dinjens WN, van Leerdam ME. Double somatic mutations in mismatch repair genes are more frequent in colorectal cancer after Hodgkin lymphoma treatment. *Gut.* 2016

Kluin PM, Langerak AW, Beverdam-Vincent J, **Geurts-Giele WR**, Visser L, Rutgers B, Schuuring E, Van Baarlen J, Lam KH, Seldenrijk K, Kibbelaar RE, de Wit P, Diepstra A, Rosati S, van Noesel MM, Zwaan CM, Hunting JC, Hoogendoorn M, van der Gaag EJ, van Esser JW, de Bont E, Kluin-Nelemans HC, Winter RH, Lo Ten Foe JR, van der Zanden AG. Paediatric nodal marginal zone B-cell lymphadenopathy of the neck: a Haemophilus influenzae-driven immune disorder? *J Pathol*. 2015;236:302-14.

Curriculum Vitae

Ina Geurts-Giele werd op 8 september 1983 geboren te Terneuzen. In 2001 behaalde ze haar VWO diploma met profielen 'Natuur en gezondheid' en 'Natuur en techniek' aan het Zeldenrust-Steelant college te Terneuzen. In september 2003 startte ze als eerste lichting met de Bachelor opleiding Psychobiologie aan de Universiteit van Amsterdam. Aansluitend volgde ze de Master Biomedical Sciences, met als richting Neurobiology. In het kader van deze opleiding liep ze wetenschappelijke stages bij de Universiteit van Amsterdam en bij het Nederlands Herseninstituut (NIN), en in 2008 behaalde ze haar diploma (cum laude). Na een korte uitstap naar een promotietraject aan het VUmc, startte Ina in maart 2009 als analist moleculaire diagnostiek op het lab van Dr. Winand Dinjens om meer praktijkervaring op te doen. Vanaf september 2010 combineerde ze deze functie met het verrichten van promotieonderzoek en het pro forma volgen van de opleiding tot Klinisch Moleculair Bioloog in de Pathologie (KMBP), welke sinds 2013 erkend is. Vanaf 2012 is ze fulltime bezig met zowel promotieonderzoek als opleiding. Daarnaast is ze sinds 2015 medeverantwoordelijk voor de moleculaire diagnostiek van de afdeling pathologie. Eind 2016 hoopt ze haar opleiding tot KMBP af te ronden.

PhD Portfolio

Summary of PhD training and teaching

Name PhD student	Willemina RR Geurts-Giele
Erasmus MC Department	Pathology
Research School	Erasmus Postgraduate School Molecular Medicine (Molmed)
PhD period	2010-2016
Promotor(s)	Prof.Dr. FJ van Kemenade
Supervisor	Dr. WNM Dinjens

Activity	Year	Workload (ECTS)
1. PhD training		
General courses		
Short course English Biomedical Writing and Communication, Rotterdam	2012	2
MOLMED Adobe Photoshop and Illustrator course, Rotterdam	2013	0.3
Scientific Integrity, Rotterdam	2016	0.3
Specific courses		
MOLMED Course Molecular Medicine, Rotterdam	2010	0.7
MOLMED Course on Molecular Diagnostics, Rotterdam	2010	1
Course on the molecular pathology approach to cancer, EACR-OECI, Amsterdam	2011	1
Course on molecular pathology VUmc, Amsterdam	2012	1
Course on the molecular pathology approach to cancer, EACR-OECI Amsterdam	2013	1
Pathology course Centrum Bioscience en Diagnostiek, Leiden. Exam: 9.1	2013	1.5
Course Advances in diagnostic molecular pathology, Graz, Austria (1 day)	2015	0.5
Presentations		
Advancements in molecular diagnostics of lynch syndrome, JNI meeting, Rotterdam	2013	1
Solid tumor clonality determinations by next generation sequencing in clinical practice: a case study (early bird presentation), AMP, Phoenix	2013	1
Somatic aberrations of mismatch repair genes as a cause of microsatellite-instable cancers, RIGHT meeting, Rotterdam	2014	1
Next generation sequencing using the Ion Torrent, Clinical Genetics meeting, Rotterdam	2014	1
Solid tumor molecular clonality determinations: primary and metastasis versus 2 independent primaries, course advances in diagnostic molecular pathology, Graz	2015	1
Molecular determination of the clonal relationships between multiple tumors in *BRCA1/2*-associated breast an/or ovarian cancer patients is clinically relevant, ESP 2-day symposium for molecular biologists, Cologne	2016	1
Poster presentations		
Improving Lynch syndrome diagnostics by multiplex snapshot assays for the detection of mismatch repair gene LOH in MSI-H tumors · MOLMED molecular medicine day, Rotterdam · Insight, San Antonio · Seventh European meeting on molecular diagnostics, Scheveningen	2011	1
Patients with multiple lymphomas: recurrences or consecutive primary lymphomas? · MOLMED molecular medicine day, Rotterdam	2012	1

Molecular diagnostics of a single multifocal non-small cell lung cancer case using targeted next generation sequencing	2013	1
· Winter meeting PathSoc-NVVP, Utrecht		
· MOLMED molecular medicine day, Rotterdam		
· Dutch Pathology Society annual meeting, Zeist		
Successive B-cell lymphomas mostly reflect recurrences rather than unrelated primary lymphomas	2013	1
· Dutch Pathology Society annual meeting, Zeist		
Screening for somatic mismatch repair gene aberrations improves the molecular diagnostics of patients suspected for Lynch syndrome	2013	1
· AMP, Phoenix		
Tumor clonality determinations using targeted next generation sequencing	2013	1
· AMP, Phoenix		
(Inter)national conferences		
Insight meeting, San Antonio	2011	1
Seventh European meeting on molecular diagnostics, Scheveningen (1 day)	2011	0.5
Dutch Pathology Society annual meeting, Zeist (1 day)	2012	0.5
Satellite & Winter meeting PathSoc-NVVP, Utrecht	2013	1
Dutch Pathology Society annual meeting, Zeist	2013	1
AMP annual meeting, Phoenix	2013	1
AMP annual meeting, National Harbor	2014	1
ESP 2-day symposium for molecular biologists, Belgrade	2015	1
ESP 2-day symposium for molecular biologists, Cologne	2016	1
Other		
Internship Immunology, ErasmusMC, Rotterdam (4 months). Supervisor: Dr. AW Langerak	2011	
Internship Microbiology, Jeroen Bosch Ziekenhuis, Den Bosch (4 months). Supervisor: Dr. AJ van den Brule	2013, 2014	
External research project HPV primary screening, Jeroen Bosch Ziekenhuis, Den Bosch (8 months parttime)	2014	
2. Teaching		
Supervising practicals		
Microscopic anatomy and pathology from mouth to stomach. 1th year students medicine	2014	1
Supervising		
Celine Leenen, medical student, ErasmusMC, Rotterdam (PhD student)	2010	1
Isabelle Meijssen, oncology master, University of Maastricht (Master's thesis)	2010	3
Edward Post, research technician	2011	1
Multiple pathology residents during their molecular internship	2014-2016	1
Supervising diagnostic molecular pathology		
Pathan, Rotterdam	2010-2015	1
Amphia Ziekenhuis, Breda	2011-now	1
Other		
Multiple presentations as part of the multidisciplinary molecular diagnostics meeting, Rotterdam (thematic education for pathology residents):		
· *BRAF* analysis, tissue identification, MSI, clonality determinations	2014	1
· Desmoid fibramotosis, melanoma, *MET* exon 14 skipping	2015	1
· *MYD88* analysis, HPV analysis, introduction molecular biology	2016	1

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Stellingen

Behorende bij het proefschrift
Next Generation Diagnostic Molecular Pathology

1. Wanneer een patiënt gediagnosticeerd wordt met twee opeenvolgende lymfomen is de kans groot dat dit dezelfde entiteit betreft, zelfs na lange tijd. (*dit proefschrift*)

2. Multifocale niet-kleincellige longtumoren moeten beschouwd worden als potentieel multiple primaire tumoren. (*dit proefschrift*)

3. Voor de meeste *BRCA1* en *BRCA2* mutatiedraagsters met meerdere tumoren is routine histopathologisch onderzoek voldoende om de origine van de tumoren te bepalen. (*dit proefschrift*)

4. Targeted NGS is een geschikte methode om de clonale relatie tussen meerdere tumoren te bepalen. (*dit proefschrift*)

5. Het aantonen van somatische afwijkingen in een tumor kan helpen bij het uitsluiten van een erfelijke oorzaak. (*dit proefschrift*)

6. Moleculaire diagnostiek is waarschijnlijkheidsdiagnostiek.

7. Verrijken voor tumorcellen door middel van microdissectie komt de uiteindelijke interpretatie van de moleculaire resultaten ten goede.

8. De capaciteit voor het genereren van NGS data is vele malen groter dan ons vermogen deze data te interpreteren (Nekrutenko et al. *Nature Reviews Genetics*, 2012)

9. Kanker is geen oorlog die over 20 jaar is gewonnen. (*Hans Clevers*)

10. Een microscoop kan wel 40x vergroten, maar als je er voor de 41^{e} keer doorheen kijkt doet hij het ook nog gewoon. (*aangepast van Herman Finkers*)

11. Ik hoop maar dat er roze koeken zijn. (*Spinvis*)

Willemina RR Geurts-Giele, 2016