Thesis

PEDIGREE ANALYSIS OF PATIENTS WITH THERAPY-RELATED MYELOID NEOPLASMS REVEALS GERM-LINE MUTATIONS IN THE DNA DAMAGE RESPONSE GENES *BRCA1*, *BARD1* AND *TP53*

submitted by

Eduard Schulz

in order to attain the academic degree

Doctor of Medicine

(*Dr. medicinae universae*)

at the

Medical University of Graz

performed at the

Division of Hematology

under the supervision of

Prof. Dr. Heinz Sill

DECLARATION OF ORIGINALITY

I, hereby, declare that the following thesis has been written only by the undersigned and without any assistance from third parties. Furthermore, I confirm that no sources have been used in the preparation of this thesis other than those indicated in the thesis itself.

Eduard Schulz

Graz, 07/2011

ACKNOWLEDGMENTS

I owe my deepest gratitude to my supervisor and mentor, Heinz Sill, for giving me the chance of conducting research in his working group and the freedom to explore on my own. I thank him for his support regarding all aspects of my thesis and beyond, for his advices and for the discussions that strengthened my interest in hematology and promoted my academic career.

Karin Hiden deserves special tanks for introducing me in the basics of laboratory work and for her great help throughout the project.

I extend my regards to Angelika Rohn who helped me with the acquisition of clinical data.

I thank Jochen Geigl and Michael Speicher for critical discussions, and Verena Rupp, Peter Ulz and Christian Gülly for their direct contribution to the results.

I would also like to thank Christine Beham-Schmid for providing samples and performing histological examinations.

None of this would have been possible without my family to whom this thesis is dedicated. Their unconditional support helped me to stay focused on my study.

Finally, I thank everyone who supported me in any respect during the completion of the project, especially my colleagues at the Division of Hematology.

ABSTRACT

Background: Therapy-related myeloid neoplasms (t-MNs) are severe long-term sequelae of cytotoxic treatments for a primary, often malignant disorder accounting for 10% of all MDS/AML cases.

Aims: We hypothesized that cancer predisposition syndromes are prevalent in this cohort of patients and germ-line mutations in respective genes contribute to leukemogenesis.

Methods: A "nuclear pedigree" consisting of all first- and second degree relatives was obtained from 51 adult and pediatric index patients with t-MNs and evaluated for cancer predisposition syndromes according to established criteria. In conspicuous cases, genomic DNA from cultured skin fibroblasts of t-MN patients was analyzed for deleterious germ-line mutations by PCR, direct sequencing and MLPA, respectively. Deleterious heterozygous germ-line mutations were further assessed for loss of the wild-type allele in $CD34^+$ sorted leukemic cells by PCR, direct sequencing and SNP array, respectively.

Results: Twenty-five of 51 (49%) patients with t-MNs had a hematological malignancy and 26 (51%) a solid tumor as primary disease. Non-Hodgkin´s lymphoma (29%) and breast cancer (25%) were the most frequent primary neoplasms found in more than 50% of all patients. Twelve index patients indicated a hereditary cancer syndrome initiating a search for *BRCA1*, *BRCA2*, *BARD1*, *TP53* and *PTEN* germ-line mutations, respectively. Deleterious, heterozygous germ-line mutations were found in 5/51 (9.8%) individuals: Two in *BRCA1* (c.5251C>T; c.3112G>T), one in *BARD1* (c.1670G>C) and two in *TP53* (c.1146delA; c.849-852insGGCG). The *TP53* germ-line mutations have not been previously described and developed most likely *de novo*. SNP array revealed loss of heterozygosity (LOH) for *BRCA1* c.3112G>T and *TP53* c.849-852insGGCG germ-line mutations in sorted $CD34^+$ leukemic cells.

Conclusion: The prevalence of deleterious germ-line mutations seems to be increased in this cohort of t-MN patients. Preliminary data indicate that these mutations contribute to therapy-related leukemogenesis. Furthermore, results may have clinical implications with respect to genetic counseling of these patients and their relatives.

ZUSAMMENFASSUNG

Hintergrund: Therapie-assoziierte myeloische Neoplasien (t-MNs) sind schwerwiegende Spätfolgen einer zytotoxischen Behandlung, die etwa 10% aller MDS/AML Fälle ausmachen.

Ziele: Es wurde die Hypothese geprüft, dass Krebs-Prädispositionssyndrome in dieser Patientengruppe vorkommen und Keimbahn-Mutationen in den entsprechenden Genen zur Leukämogenese beitragen.

Methoden: Eine standardisierte Stammbaumanalyse, die alle erst- und zweitgradigen Verwandten inkludierte, wurde von 51 adulten und pädiatrischen Indexpatienten mit t-MNs erstellt. Bei Auffälligkeit wurden konstitutionelle DNA von kultivierten Hautfibroblasten auf Mutationen in den entsprechenden Genen mittels PCR, direkter Sequenzierung und MLPA analysiert. Pathogene heterozygote Keimbahn-Mutationen wurden in $CD34^+$ leukämischen Zellen auf Verlust des Wildtyp-Allels mittels PCR, direkter Sequenzierung und SNP-Array untersucht.

Resultate: 25/51 (49%) Indexpatienten hatten eine hämatologische Primärerkrankung, 26 (51%) einen soliden Tumor. Non-Hodgkin Lymphome (29%) und Brustkrebs (25%) waren die häufigsten Primärneoplasien. Zwölf Indexpatienten waren verdächtig auf ein hereditäres Krebssyndrom und wurden nach etablierten Kriterien auf Keimbahn-Mutationen in *BRCA1*, *BRCA2*, *BARD1*, *TP53* und *PTEN* getestet. Pathogene Keimbahn-Mutationen wurden in 5/51 (9,8%) Indexpatienten gefunden: Zwei in *BRCA1* (c.5251C>T; c.3112G>T), eine in *BARD1* (c.1670G>C) und zwei in *TP53* (c.1146delA; c.849-852insGGCG). Beide *TP53* Keimbahnmutationen wurden bisher nicht in der Literatur beschrieben und entstanden wahrscheinlich *de novo*. *BRCA1* c.3112G>T und *TP53* c.849-852insGGCG zeigten ein loss of heterozygosity (LOH) in $CD34^+$ leukämischen Zellen.

Fazit: Die Prävalenz von hereditären Krebs-Prädispositionssyndromen ist in dieser Kohorte von t-MN Patienten erhöht. Präliminäre Daten zeigen, dass diese Keimbahn-Mutationen zur Leukämogenese beitragen. Darüber hinaus könnten diese Ergebnisse klinische Relevanz bezüglich genetischer Beratung von Patienten mit t-MNs und deren Familien besitzen.

TABLE OF CONTENTS

1 INTRODUCTION 1

1.1 Myelodysplastic Syndromes and Acute Myeloid Leukemia 1

1.1.1 Definition 1

1.1.2 Genetics of MDS and AML 4

1.1.3 Common clinical features 7

1.1.4 Diagnosis 7

1.1.4.1 MDS 7

1.1.4.2 AML 8

1.1.5 Treatment 9

1.1.5.1 AML 9

1.1.5.2 MDS 11

1.2 Therapy-related Myeloid Neoplasms 13

1.3 Genetic Susceptibility to t-MNs 16

1.4 Purpose of the study 19

2 MATERIALS AND METHODS 20

2.1 Patients and Samples 20

2.1.1 Nuclear pedigree 20

2.1.2 Inclusion criteria for mutational analysis of candidate genes 21

2.1.3 Cell culture of fibroblasts 22

2.1.4 Isolation of $CD34^+$ Cells 24

2.2 Immunohistochemical Analysis of p53 26

2.3 Mutational Analysis 26

2.3.1 Isolation and purification of DNA and RNA 26

2.3.2 Assessing concentrations of nucleic acids 27

2.3.3 Polymerase chain reaction (PCR) and reverse transcription polymerase chain reaction (RT-PCR) 27

2.3.4 Methylation specific PCR (MSP) 29

2.3.5 Agarose gel electrophoresis 29

2.3.6 Post PCR processing 31

2.3.7 DNA sequencing 31

2.3.8 Multiplex ligation-dependent probe amplification 32
2.3.9 Single nucleotide polymorphism array analysis 34
2.3.10 Mutational analysis of *BRCA1*, *BRCA2* and *BARD1* 35
2.3.10.1 PCR (BRCA1) 35
2.3.10.2 RT-PCR (BRCA1) 36
2.3.10.3 Direct sequencing (BRCA1) 36
2.3.11 Mutational analysis of *TP53* 37
2.3.11.1 PCR and direct sequencing 37
2.3.11.2 MSP 39
2.3.12 Mutational analysis of *PTEN* 40

3 RESULTS 41

3.1 Patients and Primary Diagnoses 41

3.2 Characteristics of Index Patients tested for Germ-line Mutations 43

3.3 Pedigrees of Index Patients with Identified Germ-line Mutations 52
3.3.1 Pedigree UPN 5824 52
3.3.2 Pedigree UPN 5869 53
3.3.3 Pedigree UPN 6387 54
3.3.4 Pedigree UPN 6371 54
3.3.5 Pedigree UPN 6144 55

3.4 Analysis of Deleterious Germ-line Mutations in Leukemic Cells 56
3.4.1 Direct sequencing shows homozygosity in $CD34^+$ cells for two deleterious germ-line mutations ..
..... 56
3.4.2 SNP array identifies loss of heterozygosity of the wild-type allele 58

3.5 Hypermethylation of the Promoter is No Mechanism of Inactivation of the Wild-type Allele in Both *TP53* Germ-line Mutations 60

3.6 P53 Immunohistochemistry 62

4 DISCUSSION 64

5 REFERENCES 73

6 APPENDIX 89

This work was supported by grants of the Austrian National Bank, Land Steiermark and Leukämiehilfe Steiermark and presented at the Annual Meetings of the Austrian Society of Hematology and Oncology, Pörtschach, May 2011, and the European Hematology Association, London, June 2011.

1 INTRODUCTION

1.1 Myelodysplastic Syndromes and Acute Myeloid Leukemia

The fourth edition of the WHO "Classification of Tumors of the Hematopoietic and Lymphoid Tissues" distinguishes five major groups of myeloid diseases: Myeloproliferative neoplasms; myeloid and lymphoid neoplasms with eosinophilia and abnormalities of *PDGFRA*, *PDGFRB* or *FGFR1*; myelodysplastic/myeloproliferative neoplasms; myelodysplastic syndromes (MDS); acute myeloid leukemia (AML) and related precursor neoplasms (Vardiman et al. 2009).

This study concentrates on the therapy-related types of MDS and AML which are grouped together as therapy-related myeloid neoplasms (t-MNs) and assigned to the disease category "AML" in the current WHO classification (see 1.2 and Table 3). However, since t-MDS and t-AML share many features with their sporadic counterparts, an overview is given about the nature of these two malignant myeloid diseases.

1.1.1 Definition

MDS and AML are malignant, clonal disorders arising from hematopoietic stem- and progenitor cells (HSPCs). The threshold of distinguishing AML from MDS is a blast count of 20% or more in the patient's bone marrow.

MDS is the most common myeloid malignancy and regarded as a disease of the elderly with a mean age at presentation of 70 years and an incidence of over 20 per 100,000 per year in this age group (Tefferi, Vardiman 2009). With an overall incidence of 2 to 3 per 100,000, AML appears to be rare but analyzing age specific groups reveals an annual incidence between 15 and 20 per 100,000 in a population older than 70 years of age (Lowenberg, Downing & Burnett 1999, Juliusson et al. 2009). Overall, the frequency of both, MDS and AML, is rising because of ageing population.

The etiology of both, MDS and AML, is mainly unknown but the propensity of emergence at an older age suggests accumulated genetic damage to HSPCs which in some cases could be augmented by inherited susceptibility (Corey et al. 2007). With the exception of rare hereditary syndromes and cytotoxic treatments (see 1.2) as well as occupational chemical exposures like benzene contributing to the risk of myeloid

malignancy development, no other risk or true causative factors have been identified so far.

MDS is characterized by dysplasia in one or more myeloid lineages, suppression of normal hematopoiesis and increased apoptosis. It is a heterogeneous disease with several morphologic subtypes evolving from very primitive hematopoietic stem cells (HSCs) (Corey et al. 2007). These subtypes are acknowledged by the recent WHO classification (see Table 1) which also have prognostic significance (Vardiman et al. 2009). In general, the more myeloid lineages are being involved, and the higher the bone marrow blast count, the worse is the patient's prognosis. A higher blast count confers a higher risk of transformation to AML, and RAEB-2 (see Table 1) has the highest risk of transformation to AML (Malcovati et al. 2005).

Table 1. WHO classification of myelodysplastic syndromes (MDS) (Vardiman et al. 2009, Tefferi, Vardiman 2009)

Refractory cytopenia with unilineage dysplasia (dyplasia in ≥10% of myeloid cells)
Refractory anemia (ring sideroblasts <15% of erythroid precursors)
Refractory neutropenia
Refractory thrombocytopenia
Refractory anemia with ring sideroblasts
(dysplasia limited to erythroid lineage and ring sideroblasts ≥15% of bone
marrow erythroid precursors)
Refractory cytopenia with multilineage dysplasia (regardless of ring sideroblast count)
Refractory anemia with excess blasts (RAEB)
RAEB-1 (2–4% circulating blasts or 5–9% marrow blasts)
RAEB-2 (5–19% circulating blasts or 10–19% marrow blasts or Auer rods present)
Myelodysplastic syndrome with isolated del(5q)
Myelodysplastic syndrome, unclassifiable
Childhood myelodysplastic syndrome
Provisional entity: refractory cytopenia of childhood

With the exception of secondary AML transformed from MDS, *de novo* AML probably develops from a more advanced HSC or – at least in some cases – a committed progentior cell. Characteristics are lineage-restricted differentiation block and decreased apoptosis (Corey et al. 2007). Two classification systems of AML are in use. The older revised French-American-British (FAB) classification (see Table 2) is based on the morphologic appearance of the myeloid cells but - with the exception of acute promyelocytic leukemia (APL) – does not have any significance other than a descriptive one (Bennett et al. 1985). On the contrary, the current WHO classification (see Table 3) recognizes the prognostic significance of cytogenetic as well as molecular aberrations and stratifies AML entities according to recurrent genetic abnormalities. Those FAB subtypes without recurrent genetic abnormalities, i.e. with a normal karyotype, are implemented as "AML, not otherwise specified".

Table 2. Revised FAB classification of acute myeloid leukemia (Bennett et al. 1985)

Type	Name
M0	Minimally differentiated acute myeloblastic leukemia
M1	Acute myeloblastic leukemia, without maturation
M2	Acute myeloblastic leukemia, with granulocytic maturation
M3	Promyelocytic, or acute promyelocytic leukemia (APL)
M4	Acute myelomonocytic leukemia
M4eo	Myelomonocytic leukemia associated with bone marrow eosinophilia
M5a	Acute monoblastic leukemia
M5b	Acute monocytic leukemia
M6a	Erythroleukemia
M6b	Pure erythroid leukemia
M7	Acute megakaryoblastic leukemia

Table 3. WHO classification of acute myeloid leukemia and related neoplasms (Vardiman et al. 2009)

Acute myeloid leukemia with recurrent genetic abnormalities
AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1*
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
APL with t(15;17)(q22;q12); *PML-RARA*
AML with t(9;11)(p22;q23); *MLLT3-MLL*
AML with t(6;9)(p23;q34); *DEK-NUP214*
AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1*
AML (megakaryoblastic) with t(1;22)(p13;q13); *RBM15-MKL1*
Provisional entity: AML with mutated NPM1
Provisional entity: AML with mutated CEBPA
Acute myeloid leukemia with myelodysplasia-related changes
Therapy-related myeloid neoplasms
Acute myeloid leukemia, not otherwise specified

1.1.2 Genetics of MDS and AML

Genomic instability is a hallmark of cancer (Hanahan, Weinberg 2011) and, indeed, MDS and AML show somatic abnormalities on the cytogenetic and molecular level which partially overlap in these myeloid disorders. Application of single nucleotide polymorphism (SNP) arrays in MDS and AML revealed copy number alterations previously unknown by conventional cytogenetics and possibly constitutes an additional, possible prognostic stratification tool (Tiu et al. 2011, Tiu et al. 2009). In addition to genetic abnormalities, it became also apparent that there were distinct epigenetic changes with prognostic impact (Figueroa et al. 2009, Bullinger et al. 2010, Shen et al. 2010).

More than fifty percent of MDS cases reveal clonal cytogenetic abnormalities of their neoplastic cells mostly involving structural aberrations. Of these, -5/del(5q), -7/ del(7q) and +8 are the most frequent ones and comprise more than two third of all chromosomal aberrations in MDS (Haase et al. 2007). In AML, approximately 40 % of patients can be expected to have a normal karyotype and cytogenetic aberrations involving chromosomes 5 and 7 are found more frequently in secondary than in *de novo* AML. Latter are often associated with a complex karyotype and a poor prognosis (see

Table 4) (Rice et al. 2011). In contrast to MDS, balanced recurrent translocations are also observed in AML (see Table 3) comprising own disease entities (Vardiman et al. 2009, Grimwade, Hills 2009). They result in the generation of chimeric fusion proteins some of them functioning as transcriptional regulators. These include *RUNX1-RUNX1T1* in t(8;21), *CBFB–MYH11* in inv(16) and *PML–RARA* in t(15;17). Their main biological effect is inhibition of differentiation by repressing the transcriptional activity of the original transcription factor (Rice et al. 2011).

Because of its heterogeneity, identifying molecular alterations involved in the pathogenesis of MDS has been difficult so far but single-nucleotide-polymorphism (SNP) arrays were helpful in the identification of potentially causative genes in commonly deleted regions (CDR) of MDS. In del(5q) MDS *RPS14* and *SPARC* were found to be pathogenetically involved (Ebert et al. 2008, Pellagatti et al. 2007). Both genes do not show point mutations but are pathogenetic towards MDS development due to a haploinsufficient effect. Reduced expression of *RPS14* leads to increased apoptosis, disturbed erythropoiesis and macrocytosis. Induction of SPARC by lenalidomide is proposed to be one of the therapeutic effects of this agent in patients with del(5q). Furthermore, not only coding genes, but also the non coding microRNAs miR-145 and miR-146a, identified in the CDR of del(5q), were shown to be able to induce MDS specific features like neutropenia and megakaryocytic dysplasia following knockdown in mice (Starczynowski et al. 2010). Taken together, insufficiency of RPS14, miR-145 and miR-146a explain many of the morphologic features of MDS with isolated del(5q) but in other MDS subtypes it is unknown which mutations are true driver and which are only passenger mutations. The most prominent gene associated with del(7q) CDR is *EZH2* coding for a histone methyltransferase and being found as a loss-of-function mutation in 6% of MDS cases (Nikoloski et al. 2010). To date, no genetic aberrations have been found in CDRs of other common cytogenetic aberrations in MDS like +8 or del(20q).

SNP arrays also enabled the identification of mutations in *TET2*, and implementation of whole genome sequencing techniques in AML identified mutations in *IDH1* and its mitochondrial homologue *IDH2* as well as in *DNMT3A,* all of which can be found in both, MDS and AML, underlining a certain overlap and possibly common origin of these myeloid diseases (Langemeijer et al. 2009, Thol et al. 2010, Walter et al. 2011).

EZH2, *TET2*, *DNMT3A* and *IDH1/2* are directly or indirectly involved in epigenetic modification which is increasingly recognized as an important mechanism contributing to leukemogenesis (Chen, Odenike & Rowley 2010). Whereas aforementioned EZH2 regulates transcription on the coarser histone level, TET2 and DNMT3A regulate transcription on the nucleotide level. DNMT3A methylates cytosines in CpG islands and TET2 catalyzes the hydroxylation of 5-methylcytosine (Walter et al. 2011, Figueroa et al. 2010). *TET2* loss-of-function mutations are common in MDS (19 - 26%) and in secondary AML (24%) (Langemeijer et al. 2009, Delhommeau et al. 2009). *DNMT3A* mutations were frequently found in AML of subtypes M4 (13%) and M5 (20.5%) but also in 8% of MDS cases (Walter et al. 2011, Yan et al. 2011). *IDH* mutations were found with a frequency of 3.6% in MDS, 15% in secondary AML and 16% in *de novo* AML (Thol et al. 2010, Paschka et al. 2010, Pichler et al. 2011). Since *IDH* mutations have never been found together with *TET2* mutations, a common pathogenetic mechanism was sought. It has been recently shown that mutated *IDH1/2* coding for isocytrate dehydrogenases lead to similar epigenetic patterns like *TET2*. Mutated isocytrate dehydrogenases perpetuate the conversion of alpha-ketoglutarate to 2-hydroxyglutarate thereby disrupting the catalytic activity of TET2 because its function is dependent on alpha-ketoglutarate (Figueroa et al. 2010).

In addition to single gene mutations leading to altered epigenetic modifications, it has been noticed that fusion proteins such as RUNX1–RUNX1T1, CBFB–MYH11 and PML–RARA are able to induce epigenetic changes by recruiting transcriptional co-repressor complexes, histone deacetylases and DNA methyltransferases or by disruption of histone acetyltransferases. The net result is a remodeling of the chromatin structure with broad activation or repression of gene expression (Chen, Odenike & Rowley 2010). Overall, it is difficult to estimate the functional consequences of altered epigenetic states because epigenetic changes affect the genome on many levels and may increase as well as decrease expression of many target genes which in turn have their own separate effects.

Classically, genes implicated in cancer are distinguished in tumor suppressor genes and in proto-oncogenes. In order that biological effects occur, both alleles of a tumor suppressor must be affected whereas proto-oncogenes need only one hit to become true oncogenes with malignant potential (Hanahan, Weinberg 2011). Important oncogenes in

AML include the family of receptor tyrosine kinases (RTK) like *FLT3* where mutations are found in 30-40% of all AML, and *KIT* as well as the *RAS* super family of signal transducers with activating mutations in approximately 25% of AML cases (Rice et al. 2011). Activation of RTK pathways is considered a characteristic feature of AML rather than MDS and one cause of the inappropriate proliferation of myeloid clones (Rice et al. 2011). The best documented role of a tumor suppressor in MDS and AML is *TP53* but mutations occur less frequently as compared to solid cancers. *TP53* alterations are associated with MDS and secondary AML presenting a complex karyotype and abnormalities of chromosomes 5 and/or 7 (Jasek et al. 2010).

1.1.3 Common clinical features

AML and MDS share similar clinical features resulting from a suppression of normal hematopoiesis. Clinical symptoms are consequences of anemia, neutropenia and thrombocytopenia which include fatigue, fever and spontaneous bleeding diathesis. Additional symptoms of tissue infiltration – i.e. bone pain, generalized lymphadenopathy, spleno- and hepatomegaly as well as central nervous system manifestations can also occur in AML but are more characteristic of acute lymphoblastic leukemia (ALL). The number of leukemic cells ("blasts") in the peripheral blood is highly variable (0 to more than 10^9/L) and, therefore, not a constant diagnostic criteria. In summary, signs and symptoms are primarily unspecific for both entities making diagnosis challenging in some cases (Kumar et al. 2010).

1.1.4 Diagnosis

1.1.4.1 MDS

To achieve a firm diagnosis of MDS, a standard diagnostic approach is needed. It consists of examination of peripheral blood and bone marrow smears (which are the most import procedures), bone marrow histology with or without immunohistochemical staining as well as cytogenetic studies. These investigations can be complemented by molecular typing and flow cytometry if needed (Valent et al. 2007).

MDS is a diagnosis per exclusion, and minimal diagnostic consensus criteria were established to assist in making the diagnosis in less obvious situations (Valent et al. 2007). Other potential clonal and non-clonal causes of hematopoietic insufficiency and dysplasia

- including deficiencies of vitamin B_{12} and folate or viral infections like parvovirus B1, for instance - have to be ruled out first. To establish the diagnosis of MDS, cytopenia in one or more myeloid lineages, i.e. hemoglobin <10 g/L, neutrophils <1800 /L or platelets <100,000 /L, has to be persistent for at least six months unless cytogenetic studies reveal MDS specific aberrations (see Table 5) (Tefferi, Vardiman 2009). Additionally, either morphologic dysplasia in at least 10% in any of the myeloid lineage cells or a blast cell count of 5 to 19% must be present (Valent et al. 2007).

1.1.4.2 AML

Diagnosis of AML is established with the same diagnostic procedures applied in MDS. However, immunophenotyping of blast cells and molecular studies are mandatory for proper subclassification. Conventional cytogenetics should be supplemented by fluorescence in situ hybridization (FISH) in ambiguous cases or if conventional analyses fail. Since AML clones, in contrast to MDS, frequently express genetic mutations of prognostic significance (see Table 4), implementation of molecular analyses is necessary. Therefore, evaluation of *NPM1*, *CEBPA*, and *FLT3* mutations is strongly recommended in patients with cytogenetically normal AML. Except for AML with recurrent genetic abnormalities - t(8;21)(q22;q22), inv(16)(p13.1q22), t(16;16)(p13.1;q22) and t(15;17)(q22;q12) - diagnosis requires a marrow blast count of equal or more than 20% (Doehner et al. 2010).

1.1.5 Treatment

1.1.5.1 AML

In AML, the most important factor influencing treatment decision as well as prognosis is the patient's fitness represented by chronological age at its simplest. Patients younger than sixty years can expect a complete remission (CR) rate of 70 to 80% and a probability of cure of 40 to 45%. On the contrary, CR rate in patients older than sixty years is 40 to 65% but 85% will eventually relapse (Burnett, Wetzler & Lowenberg 2011). CR is defined by recovery of peripheral blood counts and a bone marrow blast infiltration below 5% (Cheson et al. 2003).

Treatment of AML is basically divided into two different courses. First, to achieve CR, a so called induction therapy is administered. Then, to reduce the risk of relapse further post remission treatment depending on the patient's prognostic risk group (see Table 4) is needed. The combination of cytarabine with an anthracycline, usually daunorubicin, is the standard induction therapy for more than 40 years, and adding a third drug could not show any significant benefit. Consolidation of CR can be achieved by several chemotherapy schedules but single high-dose cytarabine is the most widely used approach because, again, no other cytotoxic drug combinations have demonstrated significant differences in overall survival (OS) (Burnett, Wetzler & Lowenberg 2011). Furthermore, postinduction therapy is strongly influenced by cytogenetic as well as molecular information since both have prognostic significance. In patients with favorable genetic alterations - patients with APL, core-binding factor leukemias or karyotypically normal AML with *NPM1* or *CEBPA* mutations - several cycles of consolidation therapy might be sufficient to achieve a cure, and risk of treatment related mortality (TRM) of hematopoietic stem cell transplantation (HSCT) is actually higher than the relapse rate. On the other hand, because of increasing relapse rates, patients belonging to intermediate or poor prognostic risk groups have better OS rates with allogeneic HSCT compared to conventional consolidation therapy alone. Since myeloablative conditioning before HSCT is associated with high TRM rates in older adults and older adults tend to present with unfavorable cytogenetics, OS in these patients is especially poor with a five-year survival of less than 5% (Rowe, Tallman 2010). Alternative strategies in elderly (or non-fit) patients include usage of lower doses of cytotoxic agents during induction as well

as consolidation therapy, reduced intensity conditioning (RIC) regimens before HSCT, 5-azacytidine for AML with multilineage dysplasia and mere best supportive care in cases of present contraindications or co-morbidities (Rowe, Tallman 2010).

In terms of treatment approach and prognosis, APL constitutes an exception. An optimized synergistic treatment consisting of an anthracycline agent in combination with all-*trans* retinoic acid (ATRA) and white arsenic (ATO, As_2O_3) achieves CR rates of 90% to 95% and a 5-year disease free survival (DFS) of 74% while significantly decreasing toxicity (Wang, Chen 2008). Such treatment successes are not yet in sight for other AML entities.

Table 4. Prognostic groups in AML according to cytogenetics and further subdivision by specific mutations (Rice et al. 2011)

AML Category	Cytogenetics	Predominant anomalies
Favorable prognosis	t(15;17); *PML-RARA*	**Poor prognosis**
	t(8;21); *RUNX1-RUNX1T1*	*KIT*
	inv(16); *CBFB-MYH11*	**Uncertain prognosis**
		KRAS, NRAS, CBL, JAK2, FLT3-TKD
Intermediate prognosis	normal karyotype	**Favorable prognosis**
	+8	*NPM1* (no *FLT3*-ITD)
	+21	*CEBPA* (biallelic, no *FLT3*-ITD)
	+22	**Poor prognosis**
	11q23	*FLT3*-ITD
		MLL-PTD
		WT1
		RUNX1
		DNMT3A
		Uncertain prognosis
		KRAS, NRAS, IDH1, IDH2, ASXL1, FLT3-TKD
Poor prognosis	complex karyotype (> 4 abn.)	**Poor prognosis**
	-5/ del(5q)	*TP53*
	-7/ del(7q)	*EVI* overexpression
	3q abn.	

abn., abnormalities; ITD, internal tandem duplication; PTD, partial tandem duplication; TKD, tyrosine kinase domain

1.1.5.2 MDS

Before the initiation of treatment, estimation of patients' clinical outcome and OS is required to provide an optimal risk adapted strategy of MDS. Similar to AML, cytogenetics is a major prognostic factor (Haase et al. 2007). Additionally, since MDS is a very heterogeneous disease, further discrimination by age, bone marrow morphology and clinical parameters - e.g. red blood cell [RBC] transfusion dependency and serum level of LDH – is possible. To assess prognosis, two validated prognostic scoring systems are available: the International Prognostic Scoring System (IPSS; see Table 5) and the WHO adapted Prognostic Scoring System (WPSS) (Greenberg et al. 1997). The latter is a modified version of the former and additionally reflects the finding that transfusion dependency correlates negatively with patients' survival (Malcovati et al. 2005).

Table 5. The International Prognostic Scoring System (IPSS) (modified from Greenberg *et al.*1997)

	Score			
Parameter	0	0.5	1	1.5
Blasts (%)	<5	5-10	-	11-20
Karyotype	normal, del(5q), del(20), -y		complex (>3 aberrations), aberations of chromosome 7	
Number of cytopenias*	0 or 1	2 or 3		
	* Hb <100 g/L, platelets <100 x 10^9/L, neutrophils <1.8 x 10^9/L			
Risk group	Low risk	0	Intermediate II	1.5 - 2
	Intermediate I	0.5 - 1	High-risk	>2.5

Principally, treatment of MDS is limited and allogeneic HSCT is the only existing curative approach. As in AML, patients have to be suitable for this procedure: HSCT is reserved for relatively younger patients (<60 years) and/or for patients with relatively poorer prognosis (IPSS Intermediate II and High). Supportive care including RBC and platelet transfusions as well as proper antibiotic usage during infections is the basis of MDS management because all patients will require it at some time during their disease course. Therapeutic goals are hematological improvement in lower risk patients (IPSS Low

and Intermediate I) and prevention or delay of transformation to AML in higher risk patients (IPSS Intermediate II and High) (Greenberg 2010). Overall, median survival is 97 to 63 months for low risk patients and 26 to 11 months for high risk patients (Tefferi, Vardiman 2009).

For the subset of lower risk patients with isolated del(5q) and transfusion dependency, lenalidomide is the recommended front line therapy. In addition to the basic supportive care, those lower risk patients having relatively low erythropoietin (EPO) levels can benefit from erythroid stimulating agents (human recombinant EPO), also in combination with low doses of granulocyte colony-stimulating factors (G-CSF), leading to reduced RBC transfusion needs. Standard application of G-CSF is mainly indicated for neutropenic patients. Iron chelation with deferasirox or deferoxamine should be considered in lower risk patients who will probably receive more than twenty RBC units but prospective randomized trials showing an OS benefit are lacking to date (Greenberg 2010).

Higher risk patients who are not eligible for HSCT are preferably treated with hypomethylating agents (5-azacytidine, 5-aza-deoxycytidine [decitabine]) leading to a significant longer progression free survival as compared to best supportive care (WijerMans et al. 2008). On the contrary, OS is not significantly prolonged when hypomethylating agents are compared to intensive conventional chemotherapy, i.e. induction therapy consisting of cytarabine with an anthracycline. Therefore, hypomethylating agents can also be a legitimate bridge to HSCT. As in AML, RIC is an increasingly used option for elderly patients but evaluation of benefit is still ongoing (Greenberg 2010).

1.2 Therapy-related Myeloid Neoplasms

Therapy-related myeloid neoplasms (t-MNs) encompass a clinical syndrome of severe long-term sequelae following cytotoxic treatments, i.e. chemotherapy and/or radiotherapy, for a primary malignant disease or an autoimmune disorder (Vardiman et al. 2009, Vardiman et al. 2008). Although regarded as one clinical syndrome, subdivision into three subtypes of t-MNs - t-AML, t-MDS and t-MDS/MPN - is still made for practical reasons in the clinical setting. However, blast crisis of chronic myeloid leukemia is excluded from t-MNs because natural disease progression cannot be precisely delimited from *de novo* t-MN development after cytotoxic treatment (Yin, Medeiros & Bueso-Ramos 2010).

t-MNs account for roughly 10% of all cases of AML, MDS and MDS/MPN and - depending on dose and type of therapy - develop after a median latency of approximately 60 months from the initiation of the cytotoxic treatment for the primary disease to the diagnosis of respective t-MN (Smith et al. 2003b, Mauritzson et al. 2002). Basically, t-MNs present with the same signs and symptoms as their *de novo* counterparts and require equal diagnostic procedures as well as similar therapeutic strategies. The distinction between t-MNs and their *de novo* counterparts is based on the patient´s history of an antecedent cytotoxic treatment. Nevertheless, t-MNs tend to show more high-risk cytogenetics but yet are also associated with poorer prognosis if the same cytogenetic risk groups are compared against each other. (Smith et al. 2003b, Kern et al. 2004, Kayser et al. 2011). There is a lack of large prospective studies but unselected retrospective cohort studies of t-MN patients showed that the median life expectancy from diagnosis was eight to ten months (Kern et al. 2004, Smith et al. 2003a). For instance, a more recent retrospective large cohort study compared 200 patients with t-AML against 2653 *de novo* AML cases and demonstrated that the four year OS rates were significant lower in t-AML patients: 25.5% (95%-CI, 19.6-33.1%) for t-AML and 37.9% (95%-CI, 36.0-40.0%; p=0.001) for *de novo* AML patients (Kayser et al. 2011). Since no prospective randomized studies investigating treatments of t-MNs exist, standard therapy according to age and cytogenetic risk group is recommended (Rowe, Tallman 2010).

Principally, t-MNs can develop after a variety of chemotherapeutic agents (see Table 6) but two associations between the type of cytotoxic regimen and cytogenetic/

morphologic presentation as well as disease evolution are striking (Smith et al. 2003b, Mauritzson et al. 2002, Sill et al. 2011). Patients who received alkylating agents and/or radiotherapy tend to present with multilineage t-MDS after a latency period of five to ten years and to show aberrations involving chromosomes 5 and 7 or a complex karyotype. On the contrary, patients treated with topoisomerase II inhibitors tend to have shorter latency periods of one to five years and present with overt acute leukemia associated with balanced chromosomal rearrangements involving 11q23 (*MLL*) and 21q22 (*RUNX1*). This category constitutes about 20% to 30% of patients with t-MNs and predominantly exhibits a monocytic or myelomonocytic phenotype. Anyhow, since most patients receive mixed chemotherapy regimens nowadays, a consistent classification into alkylating agent or topoisomerase-II-inhibitor associated t-MNs is not regularly possible. That is why the current WHO classification does not subdivide t-MNs according to the type of previous cytotoxic treatment (Vardiman et al. 2009).

As a result of improved treatments and early detection, the number of cancer survivors increases steadily: A recent evaluation of Surveillance, Epidemiology, and End Results (SEER) data by the Centers for Disease Control and Prevention (CDC) showed that the prevalence of cancer survivors in the USA – excluding in situ cancer or non-melanoma skin cancer - rose from 3.5% (9.8 million) in 2001 to 3.9% (11.7 million) in 2007 (Centers for Disease Control and Prevention (CDC) 2011). Given the fact that cytotoxic treatment and a sufficient latency period correlate with t-MN development one must assume that the number of patients with t-MNs is likely to rise parallel to the increase in the prevalence of cancer survivors with a respective time shift. Thus, t-MNs constitute a growing health problem so that prevention and alternative therapy strategies will be of outstanding importance.

Table 6. Classification of chemotherapeutic agents by mechanism of action (Smith et al. 2003b, Kayser et al. 2011, Sill et al. 2011)

Mechanism of action/ substance group	Agent
Alkylating agents	
Nitrogen mustard	Chlorambucil, cyclophosphamide, ifosfamide, melphalan
Nitrosourea	Carmustine, lomustine
Platinum-based	Carboplatin, cisplatin, oxaliplatin
Alkylsulfonate	Busulfan, treosulfan
Hydrazine	Procarbazine
Triazene	Dacarbazine
Aziridine	Dacarbazine
Antimetabolites	
Folic acid	Methotrexate
Purine antagonist	Cladribine, clofarabine, fludarabine, mercaptopurine
Pyrimidine antagonist	Cytarabine, decitabine, azacitidine, fluorouracil, gemcitabine
Antitubulin	
Taxane	Docetaxel, paclitaxel
Vinca alkaloid	Vinblastine, vincristine, vindesine, vinorelbine
Topoisomerase II inhibitors	
Epipodophyllotoxin	Etoposide, teniposide
Intercalating agents	
Anthracycline	Daunorubicin, doxorubicin, epirubicin, idarubicin
Anthracenedione	Mitoxantrone
Streptomyces	Actinomycin, bleomycin, mitomycin

1.3 Genetic Susceptibility to t-MNs

A legitimate question is what predisposes certain individuals to the development of t-MNs. To date, a final answer cannot be given but there is growing evidence from human as well as animal studies arguing that a proportion of patients with t-MNs could have a genetic susceptibility for therapy-related leukemogenesis (Sill et al. 2011). Based on the current knowledge, this genetic susceptibility has been shown to be either syndrome associated or to be associated with genetic variations in genes involved in DNA damage repair and/or drug metabolism. Additionally, true, pure familial leukemia cases developing on the ground of *RUNX1* or *CEBPA* germ-line mutations have been described but their significance is rather negligible for the majority of patients due to extreme rarity (Owen, Barnett & Fitzgibbon 2008, Seedhouse, Russell 2007). Nevertheless, from a scientific point of view even rare hereditary diseases can turn out to be useful in the investigation of additional driver mutations of sporadic and therapy-related myeloid malignancies.

A multitude of syndromes is associated with an increased incidence of MDS and AML - including the bone marrow failure syndromes Shwachman-Diamond syndrome and severe congenital neutropenia, the DNA repair deficiency syndromes Fanconi anemia and Bloom syndrome as well as aneuploidy-associated leukemia predisposition, exemplified by Down syndrome – but only the hereditary cancer syndrome neurofibromatosis type 1 (NF1) which is caused by mutations in the tumor suppressor neurofibromin (*NF1*) and inherited in an autosomal dominant fashion could persuasively demonstrate an increased incidence of t-MNs after cytotoxic treatment in an animal model. So far, other syndromes associated with leukemia provide only preliminary evidence for an additional risk modifying role of cytotoxic therapies to the development of t-MNs (Owen, Barnett & Fitzgibbon 2008, Seedhouse, Russell 2007). A problem concerning all syndrome-associated myeloid malignancies is lack of prospective studies assessing the risk of cytotoxic therapies for the development of t-MNs in individuals with hereditary syndromes. To date, two studies, a case study by Ben-Yehuda *et al*. and a case-control study by Pagano *et al.*, reported about positive family histories of cancers in t-MN patients but no systematic evaluation of cancer spectra and respective candidate genes has been conducted yet (Ben-Yehuda et al. 1996, Pagana et al. 2001).

Individuals with NF1 are predisposed for a variety of neoplasms including myeloid malignancies, and, indeed, heterozygous *NF1*$^{+/-}$ mice develop myeloid leukemia with increased frequency compared to wild-type mice (Maris et al. 1997, Jacks et al. 1994). Furthermore, heterozygous *NF1*$^{+/-}$ mice treated with cyclophosphamide develop a myeloproliferative disorder more frequent and with shorter latency than mice not receiving the alkylating agent but etoposide or no therapy at all (Mahgoub et al. 1999). Moreover, loss of heterozygosity (LOH) at *NF1* was detected in some leukemias of these mice demonstrating the typical two hit course of tumor suppressor genes in oncogenesis (Mahgoub et al. 1999). This second hit was apparently induced by the cytotoxic cyclophosphamide administration. Similarly, radiation was also shown to be a potent inducer of a diverse spectrum of myeloid malignancies in heterozygous *NF1*$^{+/-}$ mice (Chao et al. 2005). Notably, these animal studies of *NF1*$^{+/-}$ mice were initiated by a clinical report of therapy-related MDS in patients with NF1 emphasizing the importance of rare hereditary diseases in the research of pathogenetic mechanisms (Maris et al. 1997). However, genetic analyses of bone marrow specimens showed neither LOH at the *NF1* locus nor activating *NRAS* and *KRAS* mutations raising the suspicion of *NF1* haploinsufficiency (Flotho et al. 2007). Although *NF1* haploinsufficiency is a potential mechanism of disease initiation, the possibility of uniparental disomy (UPD) at the *NF1* locus was not investigated at that time and has been later demonstrated to be a characteristic mechanism in the development of juvenile myelomonocytic leukemia (JMML) in patients with NF1 (Flotho et al. 2007). Besides, different hits downstream in the RAS-MAPK pathway, the main pathway deranged in *NF1* mutation carriers, were not investigated. Nevertheless, the research on individuals with NF1 and on mice carrying *NF1* mutations laid the groundwork for future studies of patients with increased hereditary cancer risk in the context of t-MNs.

The Li-Fraumeni syndrome (LFS) is an autosomal dominant cancer predisposition syndrome associated with germ-line mutations of the *TP53* gene and classically defined by a particular high occurrence of soft tissue and bone sarcomas, breast cancer, brain tumors and acute leukemia (Li et al. 1988, Malkin et al. 1990). Since *TP53* mutation carriers have an increased risk for the development of a variety of cancers and display

incomplete penetrance in a substantial proportion of individuals, the concept of a so called Li-Fraumeni-like (LFL) syndrome (see Table 7) emerged (Varley 2003). Several case studies reported about t-MNs in patients with confirmed *TP53* mutations but no systematic analysis has been made thus far (Felix et al. 1996, Dockhorn-Dworniczak et al. 1996, Hisada et al. 1998, Anensen et al. 2006, Talwalkar et al. 2010). According to Weitzel *et al*. leukemia is no main component of LFS/LFL, nevertheless, the relative risk (RR) of leukemia development is probably increased in *TP53* mutations carriers although the exact figure is yet unknown (Gonzalez et al. 2009). Recently, van't Veer *et al.* have evaluated 180 Dutch families referred for *TP53* mutation analysis and calculated tumor type specific cancer risks in *TP53* mutation families, *TP53*-positive and *TP53*-negative LFL families (Ruijs et al. 2010). Compared to the general population *TP53* positive families showed a non significant relative risk (RR) of 3.2 for leukemia (observed 2, expected 0.6, 95%-CI 0.4 to 12; p= 0.258) but there was a substantial trend for leukemia in *TP53* positive LFL families (RR 8.7, obs. 2, exp. 0.23, 95%-CI 1.1 to 31; p= 0.052) hinting at the need for large cohort studies of *TP53* mutations carriers. Unexpectedly, *TP53* negative LFL families had a statistically significant RR of 7.3 for leukemia (obs. 4, exp. 0.55, 95%-CI 2 to 19; p= 0.006) showing that also different genetic risk factors predispose to both, solid cancer and myeloid malignancy. During the preparation of this thesis, Link *et al*. published a report about a large deletion in the *TP53* gene identified by whole-genome sequencing in a patient with t-AML (Link et al. 2011). By demonstrating loss of the wild-type allele due to UPD in the leukemic clone they provided additional preliminary evidence for a contributing role of *TP53* germ-line mutations in the development of t-MNs.

Several studies tried to assess the associations of t-MNs with polymorphisms in genes involved in detoxification as well as DNA repair pathways but much of the data remains contradictory (Seedhouse, Russell 2007). Detoxification pathways can be roughly subdivided in phase I activation reactions generating reactive intermediates and in phase II conjugation reactions detoxifying these intermediates. Polymorphisms recurrently associated with t-MNs were found in genes coding for cytochrome p450 enzymes (phase I), glutathione S-transferase and NAD(P)H:quinone oxidoreductase (both phase II, respectively). In the same way, variants in genes involved in DNA repair pathways and shown to be repeatedly associated with t-MNs include *MSH2* (mismatch repair), *RAD51*

(double strand break repair) and *ERCC2* (nucleotide excision repair) (Seedhouse, Russell 2007, Leone et al. 2007). However, the associations between single polymorphisms and t-MNs have rather low odds ratios in the majority of cases (below 2). Therefore, Seedhouse and Russel suggested that case-control studies assessing the risk of polymorphisms to t-MNs should always include more the one gene of the same pathway (Seedhouse, Russell 2007). Consequently, they proposed a multistep therapy-related leukemogenesis model wherein altered detoxification capabilities of hematopoietic stem cells result in DNA damage during cytotoxic therapy leading to genetic instability because of impaired DNA repair mechanisms.

1.4 Purpose of the study

Given the current data and knowledge, t-MNs are in general most likely complex diseases defined by a genetic susceptibility which is characterized by multiple variants jointly leading to a pathogenic reaction to exogenous cytotoxic stress. Yet, it was hypothesized that germ-line mutations in genes causing hereditary cancer predisposition syndromes - to some extent previously unknown to be associated with t-MNs - are prevalent in a subset of t-MN patients and increase the risk of t-MN development. Hence, the present study focused on patients who received cytotoxic treatments for a malignant primary disease.

Specific aims of the present study addressed the following questions:

(1) Are hereditary cancer predisposition syndromes prevalent in patients with t-MNs?
(2) Does systematic pedigree analysis allow the identification of hereditary cancer predisposition syndromes in patients with t-MNs?
(3) Which genes exactly are involved in hereditary cancer predisposition syndromes being found in t-MN patients?
(4) Do germ-line mutations causing hereditary cancer predisposition syndromes contribute to therapy-related leukemogenesis?

2 MATERIALS AND METHODS

2.1 Patients and Samples

Sixty-nine patients were diagnosed with t-MNs and treated at the Divisions of Hematology and Pediatric Hematology and Oncology, Medical University of Graz (MUG), Graz, Austria, from February 1997 to December 2010. They or their relatives in the case of deceased patients were asked to give information about their family history emphasizing on the occurrence of cancer(s). Since this study focused on patients who developed t-MNs after cytotoxic treatments of primary malignant disorder(s), eight patients were excluded because they had developed t-MNs after treatment for an autoimmune disease. Ten more patients were not considered as they rejected participation or gave insufficient information to compile a complete nuclear pedigree (see below). Overall, fifty-one index patients with cancer as primary disease were studied. The study procedures were reviewed and approved by the institutional review board of the MUG (vote number: 19-284 ex 07/08). Written informed consent was obtained from each study participant for providing personal and family history data as well as biological specimens for molecular analyses. The morphologic subtypes of t-MNs were classified according to the latest WHO classification of myeloid neoplasms using standard techniques including morphology, immunophenotyping and molecular cytogenetics (Vardiman 2010).

2.1.1 Nuclear pedigree

A “nuclear pedigree” according to H.T. Lynch *et al.* (Lynch et al. 2008) was obtained from each patients that included at least information on the patient´s siblings, progeny and both parents (first-degree relatives), as well as maternal and paternal aunts and uncles and both sets of grandparents (second-degree relatives). Each cancer diagnosis was attempted to be confirmed through pathology reports of a medical database linking the university clinic with referral hospitals and medical reports requested from non-affiliated hospitals and family physicians. In ambiguous cases, tissue specimens were reevaluated by pathologists experienced in the diagnosis of neoplastic diseases.

2.1.2 Inclusion criteria for mutational analysis of candidate genes

To decide which patients should be tested for germ-line mutations, index cases were differentiated into three different groups.

Group A consisted of index patients who met one of the current clinical classification schemes for LFS or LFL (see Table 7). They were analyzed for *TP53* mutations only.

Table 7. Clinical criteria of LFS and LFL used as a basis for *TP53* germ-line mutation testing

Classical LFS (Li et al. 1988)	A proband with a sarcoma diagnosed before age 45 AND a first-degree relative with cancer before age 45 AND another first- or second-degree relative with either a sarcoma diagnosed at any age or any cancer diagnosed under age 45
LFS (Chompret et al. 2001)	A proband with any childhood cancer or sarcoma, brain tumor or adrenal cortical tumor under the age of 45 years AND a first or second degree relative with a typical LFS cancer at any age AND a first or second degree relative in the same lineage with any cancer under 60 years
LFS revised (Tinat et al. 2009)	A proband with a tumor belonging to the LFS tumor spectrum (soft tissue sarcoma, osteosarcoma, brain tumors, pre-menopausal breast cancer, adrenal cortical carcinoma, leukemia, lung bronchoalveolar cancer) before 46 years and at least one first or second degree relative with an LFS tumor (except breast cancer if the proband is affected by breast cancer) before 56 years or multiple primary tumors
	A proband with multiple primary tumors (except multiple breast tumors), two of which belong to the LFS tumor spectrum and the first of which occurred before 46 years
	A proband with adrenal cortical carcinoma or choroid plexus tumor, irrespective of the family history
LFL (Birch et al. 1994)	A proband with any childhood cancer or sarcoma, brain tumor or adrenal cortical tumor under the age of 45 years AND a first or second degree relative with a typical LFS cancer at any age AND a first or second degree relative in the same lineage with any cancer under 60 years
LFL (Eeles 1995)	Two different tumors that are part of extended LFS in first or second degree relatives at any age (sarcoma, breast cancer, brain tumor, leukemia, adrenal cortical tumor, melanoma, prostate cancer and pancreatic cancer)

Group B included all index patients who met the German national Step 3 guideline (Table 8), thereby having chances an over ten percent chance for germ-line mutations in *BRCA1* or *BRCA2* (Kreienberg et al. 2008). They were analyzed for mutations in *BRCA1* and *BRCA2* as well as for *BARD1* C557S. Mutational analysis of *BARD1* C557S was only performed, when no mutations in the *BRCA* genes were found. Group B index patients who had negative test results for *BRCA1* or *BRCA2* germ-line mutations were tested for *TP53* first and subsequently for *PTEN*.

Table 8. Under these conditions, chances are over 10% that germ-line mutations in *BRCA1* or *BRCA2* can be found

- Families with a breast cancer patient affected before the age of 36 years
- Families with at least two affected patients (breast or ovarian cancer), one patient younger than 51 years
- Families with three or more patients with breast or ovarian cancer
- Families with two patients with ovarian cancer
- Families with bilateral breast cancer before the age of 51 years
- Families with concurrent breast and ovarian cancer
- Families with one breast cancer patient and one ovarian cancer patient
- Families with a male breast cancer patient and a female breast or ovarian cancer

Group C included all index patients who did not meet the inclusion criteria of categories A and B but showed either multiple, i.e. two or more, primary tumors - benign neoplasms included - or a marked family history for cancer hinting at a different tumor predisposition syndrome. Group C index patients were tested for candidate genes fitting their tumor spectrum best.

2.1.3 Cell culture of fibroblasts

Collecting of tissue samples and primary tissue culture were conducted previously at the Division of Hematology. Tissue samples were obtained by punch biopsy from index patients with t-MNs before therapy for the myeloid malignancy has been commenced. Fibroblasts were isolated from these skin pieces in explant cultures and cryopreserved in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) with the addition of 10% dimethyl sulfoxide (DMSO; WAK-Chemie Medical, Steinbach, DE) in liquid

nitrogen at -196°C. In cases where a primary culture of fibroblasts was unsuccessful, skin samples were cryopreserved under the same conditions as cultured fibroblasts.

Isolated fibroblasts of t-MN patients used in this study were counted in Bürker-Türk hemocytometers before cryopreservation and after thawing or harvesting, thereby measuring the viability with Trypan blue. Thawing was conducted in a 37°C water bath. Growth medium used for cell culture consisted of DMEM with 10% fetal bovine serum (FBS; PAA, Pasching, Austria, Lot No. 6/A10408-2367/RMR5706) and 1% penicillin/streptomycin (PenStrep; Sigma-Aldrich). Cells were grown in adherent cultures in culture flasks (Costar, Corning, NY, USA) whose size was chosen to keep a cell to surface ratio of $4x10^4$ cells per square centimeter. They were processed either in a confluent state, usually after seven days of growing, or when they stopped growing at all. Culture flasks were stored in a cell incubator (models BBD6220 or BB16 function line; Heraeus instruments, Hanau, DE), at 37°C, 5% CO_2 and 95% humidity. Trypsin in EDTA (TIE; Sigma-Aldrich) was used to harvest fibroblasts adherent to the wall of the flasks. The amount of TIE was dependent on the surface size of the flasks, so that the bottom of the flask was covered entirely (10 mL/225 cm^2, 5 mL/150 cm^2, 2.5 mL/75 cm^2). The enzymatic activity of trypsin was stopped with a twofold amount of growth medium compared to TIE. To accomplish separation from solutions like growth medium or TIE fibroblasts were washed and resuspended with PBS. All necessary centrifugation steps for cell washing were carried out at 1300 rpm and 5°C for five minutes (models CS6R or Spinchron; Beckmann, USA). Harvested cells were either shock frozen in liquid nitrogen for DNA isolation and stored in a CO_2 freezer (Bio Freezer N8431, Forma Scientific, OH, USA) at -70°C or cryopreserved with 10% DMSO (WAK-Chemie Medical) in liquid nitrogen tanks (model Espace 331 Liquide; Air Liquide, Marne La Vallee Cedex, France; model 10K, Taylor-Wharton, Theodore, Al, USA) after controlled down cooling in an isopropanol box (Cryo 1°C freezing container; Nalgene Labware, Thermo Fisher Scientific, Rochester, NY, USA) for at least 4 hours at -70°C.

2.1.4 Isolation of CD34+ Cells

It is generally accepted that leukemia initiating cells (LIC) are enriched in the cell population expressing cell surface antibody CD34 but not CD38 (Lapidot et al. 1994).

To determine if leukemic blasts showed homozygosity in the gene of interest $CD34^+$ cells were isolated and genetically tested. Blood samples from patients with t-MNs at the time of diagnosis were collected previously and separated to its components by Ficoll-Paque PLUS (StemCell Technologies, Grenoble, France) according to manufacturer's protocol. Mononuclear cells (MNCs) were cryopreserved in Roswell Park Memorial Institute medium (RPMI [Sigma-Aldrich, St. Louis, MO, USA]) with 10% DMSO (Sigma-Aldrich) in liquid nitrogen as described (see 2.1.3). $CD34^+$ cells were separated from mononuclear cells after thawing by using the magnetic-activated cell sorting (MACS) CD34 MicroBead Kit together with MS Columns and MiniMACS Separator (all Miltenyi Biotec, Bergisch Gladbach, DE) according to manual of the supplier. The purity of $CD34^+$ cells was measured by flow cytometry with FACSCalibur (BD, Franklin Lakes, NJ, USA) according to manufacturer's protocols. The reagent mixture for the fluorescence-activated cell sorting (FACS) consisted of 1µL of eluted $CD34^+$ cells, 5 µL of CD38 FITC (BD), 2.5 µL of CD34 PE (BD), 5 µL of CD45 APC (BD) and 5,0 µL of 7-AAD (BD). A purity of 90% $CD34^+$ cells was considered acceptable for the appropriate analyses. DNA was isolated from CD34 positive as well as negative cells as described (see 2.3.1).

Ficoll is a hydrophilic polysaccharide with a high molecular weight of 400 kD. After density centrifugation of blood covering Ficoll, MNCs form a layer, the buffy coat, above the Ficoll reagent and can be removed easily by gentle suction with a pipette (Noble, Cutts 1967). The MNCs layer obtained from healthy individuals consists mainly of lymphocytes and monocytes, but is enriched for leukemic blasts in patients with a myeloid disorder.

The principle behind MACS separation is labeling of antigens with MicroBeads which are complexes consisting of antibodies conjugated to magnetic nanoparticles (Schmitz et al. 1994). By loading a cell suspension onto a MS Column which is placed in the magnetic field of a MiniMACS Separator, magnetically labeled cells - here monoclonal mouse anti-human CD34 antibody (mouse IgG1) labeled $CD34^+$ cells - are retained within

the column while the unlabeled cells run through. After removing the magnetic field positively separated cells can be eluted. To prevent unspecific binding of antibodies to the cell surface, one has to work on ice and use pre-cooled solutions. Furthermore, human IgG FcR Blocking Reagent (Miltenyi Biotec) is used to block unwanted binding of antibodies to human Fc receptor-expressing cells, thereby increasing the specificity of labeling with MicroBeads.

FACS is a subtype of flow cytometry based upon the light scattering of fluorescently labeled cells in a heterogeneous cell mixture flowing in a fine liquid stream (Janeway et al. 2001). Scattered light as well as emission of light after stimulation with a laser beam of a single wavelength are detected by several detectors directed in line with the laser (forward scatter) and perpendicular to it (side scatter). By direct immunofluorescent staining using an anti-human anti-CD34 antibody (mouse IgG1 CD34 PE; BD, Franklin Lakes, NJ, USA) recognizing an epitope different from that bound by the MicroBeads and counterstaining with an antibody against the Leukocyte Common Antigen (LCA) CD45 (mouse IgG1 CD45 APC; BD, USA]), $CD34^+$ cells can be discriminated from other MNC and depicted in a two dimensional dot plot after computational analysis (Craig, Foon 2008). 7-amino-actinomycin D (7-AAD) is a chemical compound with fluorescent nature and strong affinity to double-stranded DNA, especially G-C rich regions. It can intercalate with intracellular DNA if the membrane of the cell is impaired or disrupted making 7-AAD a useful viability marker (Schmid et al. 1992). Since it was aimed for the DNA of $CD34^+$ cells, viability was not an issue. A so called isotype control consisting of an anti-mouse IgG1 antibody panel labeled with the same fluorescent dyes was used as negative control. The isotype control should not bind to the eluted cells of interest.

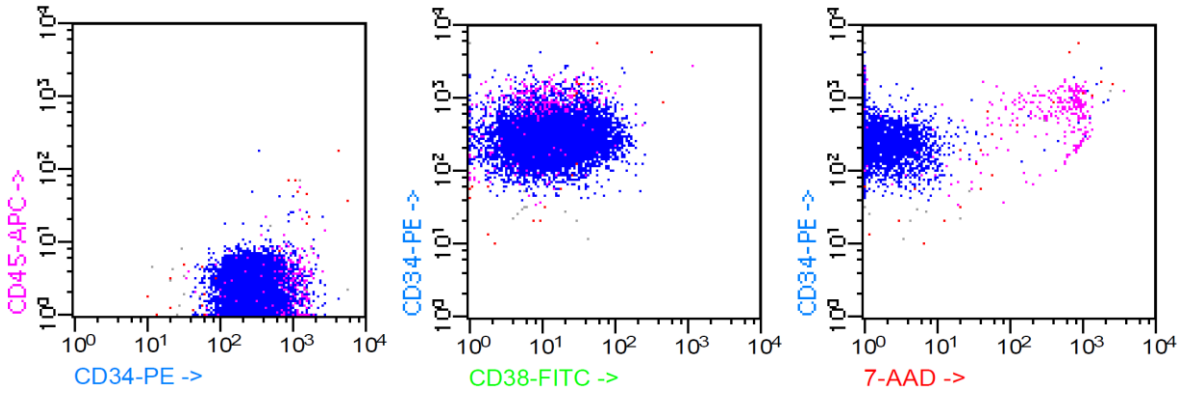

Figure 1. Representative illustration of FACS analysis of CD34$^+$ leukemic cells isolated by MACS. Cells show diminished staining for LCA (left plot) and are negative for CD38 (middle plot). The viable cells are depicted as blue events in the right plot.

2.2 Immunohistochemical Analysis of p53

Immunohistochemistry of p53 was performed on paraffin embedded tissue at the Institute of Pathology (MUG) with the IgG2b monoclonal mouse anti-human antibody clone DO-7 (M7001, DakoCytomation, Glostrup, DK) according to the recommendations of the supplier (edition 18.12.02). The epitope recognized by the antibody is located between the N-terminal amino acids 1 and 45 and possibly between amino acids 37 and 45 of the human p53 protein (Vojtesek et al. 1992).

2.3 Mutational Analysis

2.3.1 Isolation and purification of DNA and RNA

DNA was isolated and purified in one session from shock frozen fibroblasts. In cases where either a mutation confirmation in a different tissue was needed or fibroblasts were unavailable, because patients refused punch biopsy, DNA from leukocytes, granulocytes, MNCs or CD34$^+$ cells was used. Isolation and purification of DNA was performed using the QIAamp DNA Blood Mini Kit and the QIAamp DNA Mini Kit (Qiagen, Hilden, DE), respectively - both according to manufacturer's protocols. RNA was obtained from fibroblasts and CD34$^+$ leukemic cells with the RNeasy Mini Kit (Qiagen) according to the manufacturer's manual.

2.3.2 Assessing concentrations of nucleic acids

Concentration of purified DNA and RNA was calculated from optical density (OD) at 260 nm measured by DU-640 spectrophotometer in triplicate:

$$OD260_{(\text{mean of 3 measurements})} \times \text{dilutional factor} \triangleq \text{Concentration of nucleic acids (ng/µL)}.$$

The dilutional factor of 5000 equates to a 1:100 solution of purified double-stranded DNA with a concentration of 50 µg/mL. The respective dilutional factor for RNA is 4000. Ratio of absorbance at 260 and 280nm ($A_{260/280}$) was used to assess the purity of nucleic acids. A ratio of A_{260}/A_{280} higher than 1.8 was considered being adequate sample purity.

2.3.3 Polymerase chain reaction (PCR) and reverse transcription polymerase chain reaction (RT-PCR)

The polymerase chain reaction (PCR) is a laboratory technique permitting "cell-free molecular cloning" and successful amplification of "a single target molecule in a DNA background of 10^5 cells" (Saiki et al. 1988). Exponential amplification of DNA is accomplished by the use of a pair of oligonucleotide primers flanking the DNA sequence of interest. PCR runs through repeated cycles of heating and cooling.

Initially, double-stranded DNA has to be denaturated so that the hydrogen bonds between complementary bases are being released resulting in the formation of single-stranded DNA. This denaturation step requires a temperature of approximately 95°C necessitationg a thermostable DNA polymerase other than the Klenow fragment. The Klenow fragment is part of Escherichia coli DNA polymerase I resulting from cleavage by a serine endopeptidase and lacking the 5' to 3' exonuclease activity (Klenow, Henningsen 1970). Since it denaturated at the necessary DNA melting temperature as well, the Klenow fragment had to be added each new cycle. It was finally replaced with a thermostable DNA polymerase extracted from the thermophilic bacterium, Thermus aquaticus (*Taq*), making the PCR "amenable to automation" and improving "the overall performance […] by increasing the specificity, yield, sensitivity and length of targets that can be amplified" (Saiki et al. 1988).

After the denaturation step, temperature is lowered and annealing of the primers to the single-stranded template DNA occurs. Primers are synthetic oligonucleotides having a length of about 20 (18-25) nucleotides and being complementary to the 3' ends of each single strand. They are the starting points for the DNA extension by *Taq* polymerase in 5' to 3' direction. The annealing temperature is dependent on the melting temperature (Tm) of the primers. Tm denotes the temperature at which half of the primers are bound to the template. An annealing temperature below the Tm of the primers leads to insufficient primer-template hybridization, whereas a temperature above the Tm results in lower amplicon - i.e. PCR product – yield (Strachan, Read 1999b).

The last step is the actual extension of the single-stranded DNA by *Taq* polymerase. During the heating between the annealing step and the extension step, mismatched primer template interconnections are dissolved resulting in a more specific extension when *Taq* polymerase reaches its temperature optimum. *Taq* polymerase performs the incorporation of deoxynucleotide triphosphates (dNTP) into the single-stranded DNA by adding the 5'-phosphate group of the dNTP to the 3'-hydroxyl group. Hence, dNTP in addition to a special buffer solution with Magnesium as a cofactor have to be included in the reaction mixture. The fidelity of *Taq* polymerase with an estimated misincorporation rate of 2×10^{-4} per nucleotide per cycle is a disadvantage, which has to be noted when large amplicons greater than four thousand bases (4 kb) are generated (Saiki et al. 1988)(Strachan, Read 1999c). The steps of denaturation, annealing and extension are repeated in cycles but their actual length of time has to be determined empirically for each PCR.

The HotStarTaq DNA Polymerase (Quiagen, Hilden, DE) used in this study had an optimized extension temperature at 72°C according to the HotStarTaq PCR Handbook (version 2008). Furthermore, an initial activation step of 95°C for 15 minutes was needed prior to the denaturation step making working on ice to prevent early unspecific extension redundant.

A variant of PCR is the reverse transcription PCR (RT-PCR) which was also applied in this study. RT-PCR makes it possible to amplify RNA transcripts after conversion of the messenger RNA (mRNA) to complementary DNA (cDNA) with a DNA-dependent RNA

polymerase (reverse transcriptase). The reverse transcriptase uses an oligo dT primer, i.e. an oligonucleotide consisting of deoxythymidines, as a starting point for transcription from mRNA to cDNA. The oligo T primer pairs with the polyadenine (poly[A]) sequence at the 3' end of eukaryotic mRNA (Strachan, Read 1999a). The applied GeneAmp RNA PCR Kit (Applied Biosystems, Foster City, CA, USA) included the MuLV (recombinant Moloney murine leukemia virus) reverse transcriptase and 16 bases long oligo dT primer. The PCR is basically performed in the same manner as described, with primers complementing cDNA (Saiki et al. 1988).

2.3.4 Methylation specific PCR (MSP)

Methylation specific PCR (MSP) is performed to investigate whether certain regions of a gene are hypermethylated and as a result less strongly expressed (Herman et al. 1996). Bisulfate treatment of DNA changes cytosine residues, but not 5-methylcytosine residues to uracil. Bisulfate treated DNA is used in the following PCR which are carried out with both methylation-specific oligonucleotide primers binding only complementary unconverted 5-methylcytosines and unmethylated-specific oligonucleotides binding thymines converted from unmethylated cytosines, respectivly. If the region of interest is hypermethylated, amplification occurs only in the reaction with the methylation-specific primers.

2.3.5 Agarose gel electrophoresis

Size and specificity of the PCR products were controlled by agarose gel electrophoresis before direct sequencing. 10 µL of each PCR product were mixed with 6x Loading Dye (Fermentas, St. Leon-Rot, DE) to a final concentration of 2x and injected together with a DNA ladder (GeneRuler 100 bp Plus; Fermentas) as control into an agarose matrix consisting of 1-1.5% agarose polymer (Scientific, Oldendorf, DE), 0.5x TBE (5,9 g/L Tris base, 2,75 g/L boric acid, 1 mM EDTA) and 0.3µg/mL ethidium bromide. The agarose gel was covered with 0.5x TBE, shortly after it had been congealed. After application of a current at 150 V to 200 V for 30 min to 45 min the gel was put in the Gel Doc 1000 (Bio-Rad, CA, USA) and illuminated with UV light. DNA bands were visualized and analyzed digitally by Quantity one 4.0.3 (Bio-Rad).

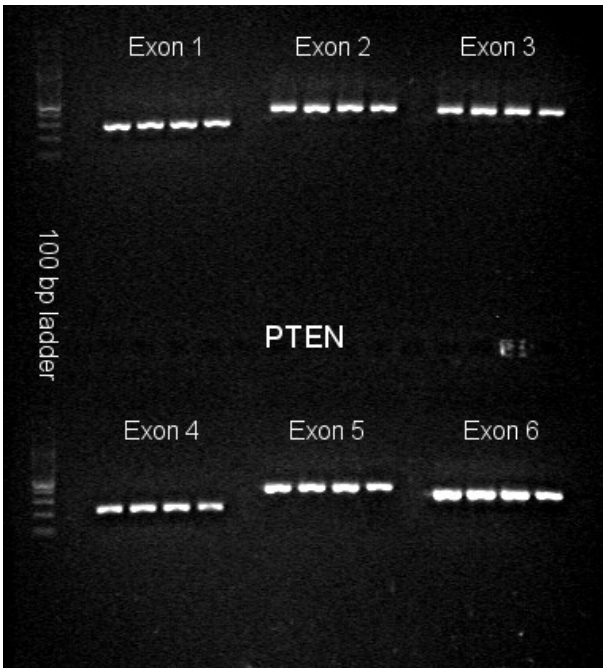

Figure 2. Representative illustration of agarose gel electrophoresis. Exons 1 to 6 of the *PTEN* gene of four different patients were amplified. bp, base pair

Agarose gel electrophoresis of DNA is performed to separate different DNA fragments by their length (Brody, Kern 2004). The method relies on the negative charged phosphate group of the nucleic acids resulting in movement of DNA towards the positively charged anode. The separation is achieved through the sieving effect of the agarose matrix which forms pores when it is solidified. A solution of Trisboric acid-disodium EDTA (TBE buffer) is used not only as conductive medium but also for the purpose of stabilization of pH above 7 so that phosphates are kept fully deprotonated. Moreover, by binding divalent cations like Mg^{2+}, which is a cofactor of nucleases, EDTA protects nucleic acids from degradation. The size of the DNA can be estimated by the addition of a reference DNA ladder which has well-defined DNA fragments. Loading buffer is injected together with the DNA solution. It contains a dye for visual tracking of DNA migration and glycerol to hold the injected solution at the bottom of the well. Ethidium bromide added to melted agarose gel intercalates with DNA during electrophoresis and makes the fragments visible after stimulation with UV light.

2.3.6 Post PCR processing

After PCR and dye-terminator reaction (see below), products were purified by Sigma Spin Post-Reaction Clean-Up Columns or Plates (SIGMA, Saint Louis, MO, USA) – according to the recommendations of the supplier - thereby removing primers, unincorporated dyes, excess salts and other interfering materials.

2.3.7 DNA sequencing

Sanger sequencing, also called chain terminating reaction, is a method to determine nucleotide sequences in DNA (Sanger, Nicklen & Coulson 1977). Instead of the usual dNTP, 2',3'-dideoxynucleoside analogues (ddNTP) are used. DdNTP lack the 3'-hydroxyl group which is needed by the DNA polymerase to extend the nucleotide chain leading to a stop of DNA extension precisely at the position of the incorporation of the ddNTP. In the original report by Sanger *et al.* four separate reaction mixtures were prepared containing only one specific ddNTP - i.e. ddATP, ddCTP, ddGTP or ddTTP, respectively - as well as the usual dNTP. Radioactively labeled dATP and dTTP were added at different points of the incubation cycles. Two small restriction enzyme fragments of the DNA of the bacteriophage ϕX174 were used as primers. DNA polymerase I of Escherichia coli was cleaved by a serine endopeptidase, thereby loosing the 5' to 3' exonuclease activity. The resulting Klenow fragment was utilized as polymerase in all reactions (Klenow, Henningsen 1970). After incubation of the four reaction mixtures, electrophoresis was performed on a denaturating acrylamide gel with each of reaction mixtures in parallel lanes. DNA sequence could be read directly from autoradiography images by comparing the relative length of the oligonucleotide bands in the individual lanes.

To achieve automated real-time DNA sequence analysis the initial chain terminating reaction was evolved by combining each ddNTP with different fluorophores (Smith et al. 1986). The resulting dye-terminator reaction, the principle behind the method used in this study, has the advantage that only one reaction mixture as well as only one single gel is needed. Furthermore, radioactive material is redundant. Each dye has a specific absorption curve and, therefore, a specific emission spectrum. During capillary electrophoresis DNA sequence is recognized by a detector measuring the different wave lengths emitted by the four different fluorophores labeled to ddNTP (see Figure 3). Since the dyes have different relative fluorescence capability, they have to be averaged in their

concentrations so that the emission curves have approximately the same height. The raw data obtained by electrophoresis has to be corrected for several factors which lead to a more difficult analysis: first, the emission spectra of the four fluorophores overlap, and, second, mobility shifts result from oligonucleotides moving at varying speeds, dependent on the ddNTP and the dyes bound to them, respectively.

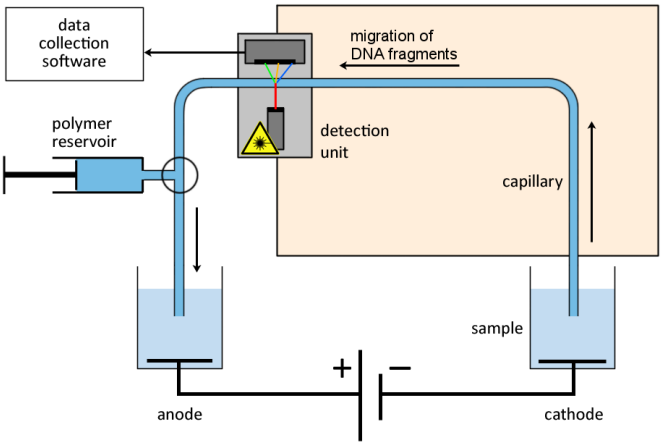

Figure 3. Capillary electrophoresis and fluorescence detection of oligonucleotide fragments (reproduced with permission of Jan Beckendorf, University of Heidelberg)

Today's dye-terminator reactions are combinations of PCR (see 2.3.3) with fluorescent modified Sanger sequencing leading to higher specificity of the sequenced DNA, because PCR products are used as templates, while the terminator reactions themselves are carried out on separate single strands with thermostable polymerases.

2.3.8 Multiplex ligation-dependent probe amplification

Multiplex ligation-dependent probe amplification (MLPA) is a PCR based technique using only one single primer pair to amplify simultaneously various probes that hybridized to target regions of interest (Schouten et al. 2002). The resulting amplification products can be analyzed by capillary electrophoresis. MLPA is especially suitable for the detection of deletions and duplications of whole exons which would otherwise be missed by direct sequencing (see 2.3.10.3) where only the unaltered allele would be analyzed. The comparison of peak patterns (see Figure 5) of control samples and test samples will show gains or losses, if the copy number is altered. The MLPA reaction runs in the following

way (see Figure 4): Each MLPA probe consists of two oligonucleotides with a hybridization sequence complementing target DNA. One is synthetic, the other one is phage M13-derived and has a unique recognizable stuffer sequence. After denaturation, oligonucleotides bind to the target DNA (1.) and are ligated by a thermostable ligase (2.). A washing step is interposed removing all unbound as well as unligated probes. Only ligated probes are then amplified by PCR with a single primer set complementing to the 5' (Y) and 3' (X) ends of the probes, respectively (3.). Each amplification product has a unique length and can be recognized by electrophoresis. Differences in relative amounts of the target sequences can be depicted in a ratio chart. Deviations of 40 percent and above compared to the reference sample are suspicious for gains or losses and should prompt verification by a different MLPA kit. Since oligonucleotides of MLPA probes are specific for target sequences, single nucleotide variations in these sequences can lead to false results.

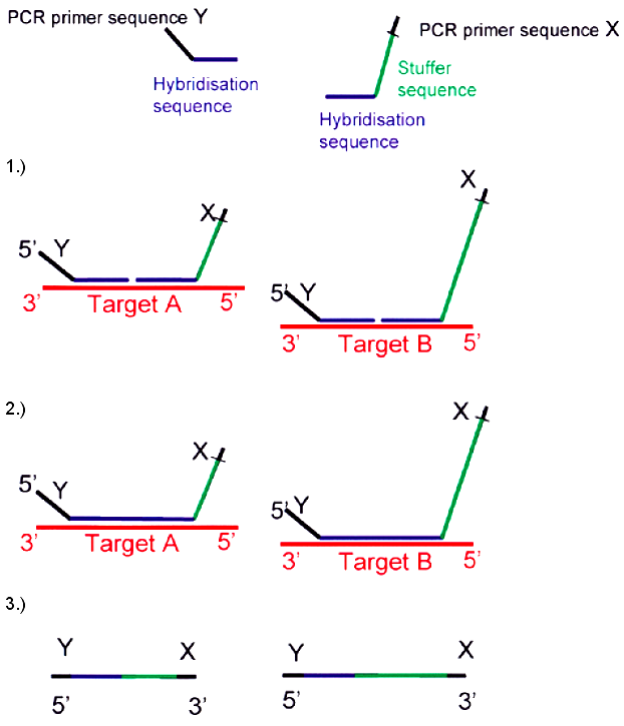

Figure 4. MLPA reaction (see text for explanation); modified from (Schouten et al. 2002)

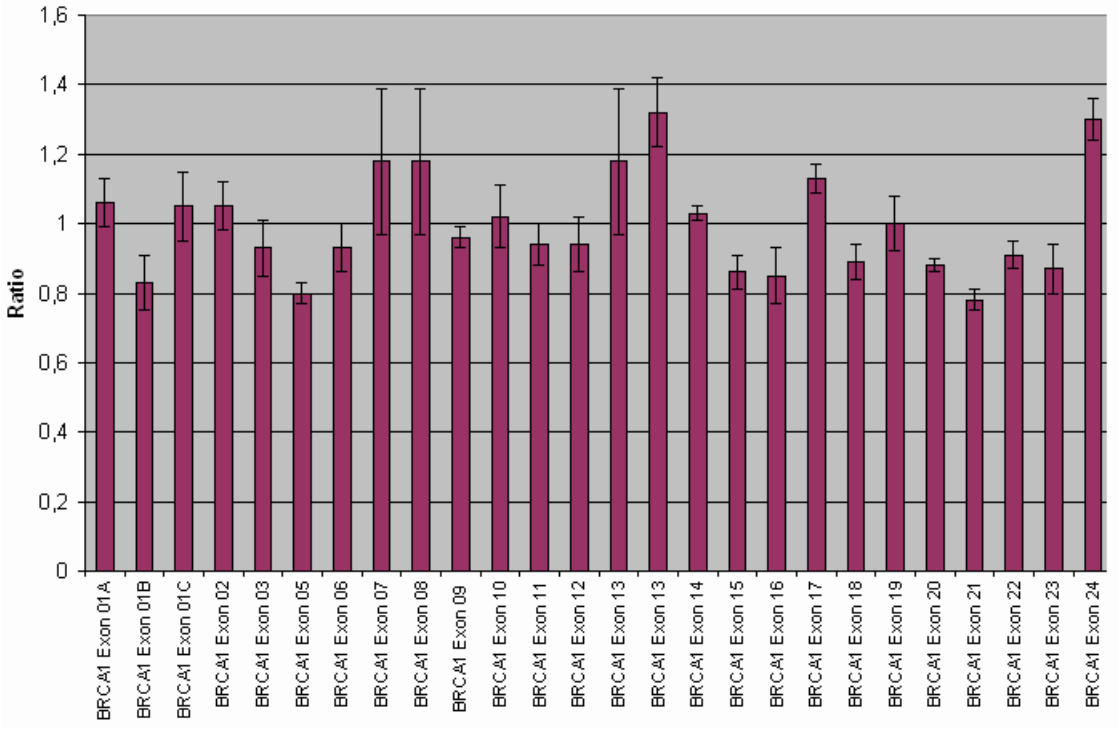

Figure 5. Representative MPLA ratio chart of patient 6353 tested for BRCA1 large genomic rearrangements (no alteration detected)

2.3.9 Single nucleotide polymorphism array analysis

Single nucleotide polymorphism (SNP) array analysis was performed to asses copy number variations (CNVs) and to distinguish loss of heterozygosity (LOH) caused by hemizygous deletion from LOH occurring after uniparental disomy (UPD). To achieve that purpose, Genome-Wide Human SNP Array 6.0 (Affymetrix, Santa Clara, CA, USA) was used according to manufacturer´s instructions. This SNP array contained about 1.8 million genetic markers roughly equally divided in probes detecting SNPs and CNVs and evenly spread over the human genome (Affymetrix 2009).

The basic rationale behind SNP array analysis is the comparison of SNP hybridization signal values of samples of interest against normalized samples (Zhao et al. 2004). Normalized samples can be matched samples from the same proband, if somatic tumor DNA is compared with constitutional DNA, or samples from a databank like the human haplotype map (HapMap) project (International HapMap Consortium et al. 2007). The

obtained raw signal data is inferred to a copy number state. Normal diploid cells have a copy number state which is equivalent to 2. CNVs like deletions correspond to a copy number state less than 2, whereas duplications show copy number states greater than 2. The detection of copy number neutral LOH due to UPD is accomplished by the finding of an unexpected large region of homozygosity within a locus showing a copy number state corresponding to 2 (Zhao et al. 2004).

2.3.10 Mutational analysis of *BRCA1*, *BRCA2* and *BARD1*

Screening for mutations in *BRCA1*, *BRCA2* and *BARD1* was conducted at the Institute of Human Genetics, Graz, Austria. The oligonucleotide primer pair used for amplification of exon 7 of the *BARD1* gene and the respective PCR program are described in Table 9. The methods of *BRCA1/2* analysis were as previously described (Engert et al. 2008). All *BRCA1* and *BRCA2* exons as well as the surrounding intronic sequences were directly sequenced. All deleterious mutations and genetic variants were named according to the BIC database (http://research.nhgri.nih.gov/bic/). To screen for LGR encompassing one or more *BRCA* exons MLPA analysis (see 2.3.8) of the *BRCA* genes was additionally performed (MLPA, SALSA MLPA KIT P002-B1 [BRCA1] and SALSA MLPA KIT P045-B1 [BRCA2], MRC Holland, Amsterdam, the Netherlands). Germ-line mutations found in primary fibroblasts were confirmed by analyzing DNA from different tissue(s) of the same individual and if possible from leukocytes of relatives.

Table 9. Oligonucleotide primer pair used for amplification of exon 7 of the *BARD1* gene.

Region amplified	Primer pairs (5' to 3')
Exon 7	AGCAGCTTTTGATTCTAGATTCTTC (fw) CACAGTAGCTAATACTCAGGAAGTGC (rv)
fw, forward primer; rv, reverse primer	
Initial denaturation at 95°C for 15 min; 35 cycles of denaturation (95°C for 45 s), annealing (57°C for 30 s), extension (72°C for 45 s); final extension time 10 min	

2.3.10.1 PCR (BRCA1)

Oligonucleotide primer sequences for mutation validation of *BRCA1* in genomic DNA were kindly provided by the Institute of Human Genetics, Graz, Austria. The sequences

of the primer sets were as follows: exon 10 (468 bp product length), 5'-ATCACTGCAGGCTTTCCT-3' (forward), 5'-CAATTTTGGCCCTCTGTTTC-3' (reverse); exon 20 (343 bp product length), 5'-CTGGCCTGAATGCCTTAAAT-3' (forward), 5'-GGTTGGGATGGAAGAGTGAA-3' (reverse). PCR was performed with 100 ng total genomic DNA, 0.3 µM of each primer (Applied Biosystems, Warrington, UK), 200 µM of each dNTP (Applied Biosystems), 1x PCR Buffer (Qiagen, Hilden, DE), additional $MgCl_2$ for a final concentration of 3 mM MG^{2+}, and 1.25 U/reaction HotStarTaq DNA Polymerase (Qiagen) in a final volume of 50 µL. A negative control (without template DNA) was included. After an initial denaturation at 95°C for 15 min, 25 cycles of denaturation (94°C for 1 min), annealing (59°C [exon20] or 57°C [exon10] for 1 min) and extension (72°C for 1 min) were performed on Mastercycler gradient (Eppendorf, Hamburg, DE), GeneAmp PCR System 9700 (Perkin Elmer Applied Biosystems, Norwalk, CT, USA) or PTC-200 (MJ Research, Watertown, MA, USA), respectively. A final extension time was performed for 10 minutes.

2.3.10.2 RT-PCR (BRCA1)

Isolated RNA was transcribed to DNA with the GeneAmp RNA PCR Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer´s manual. Primers complementing cDNA were designed with Primer-BLAST (NCBI, http://www.ncbi.nlm.nih.gov/tools/primer-blast/) to cover all six transcript variants of the BRCA1 gene. The sequences of the primer sets were as follows: exons 16 to 24 (498 bp product length), 5'-TGTCCATGGTGGTGTCTGGCC-3' (forward), 5'-TCCAGGCATCTGGCTGCACA-3' (reverse); exons 20 to 24 (271 bp product length), 5'-CCACCAAGGTCCAAAGCGAGCA-3' (forward), 5'-TCGGGTCACCACAGGTGCCT-3'(reverse). The reaction mix and the thermal cyclers were the same as described earlier (see 2.3.10.1). After an initial denaturation at 95°C for 15 min, 30 cycles of denaturation (94°C for 30 s), annealing (61°C for 30 s) and extension (72°C for 1 min) were performed. The final extension time was 10 minutes.

2.3.10.3 Direct sequencing (BRCA1)

The dye-terminator reaction was performed using the BigDye Terminator version 1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on the Mastercycler gradient (Eppendorf, Hamburg, DE) or the PTC-200 (MJ Research, Watertown, MA, USA),

respectively, according to the manufacturer´s protocol. The reaction mix consisted of 4 µL 2.5x Ready Reaction Premix, 4 µL 5x BigDye Sequencing Buffer, 0.5 µM Primer (forward and reverse separately, the same as for PCR) and 1µL of cleaned PCR product in a final volume of 20 µL. The dye-terminator PCR products were purified as described (see 2.3.6) and dissolved in 20 µL Hi-Di Formamide (Applied Biosystems, Warrington, UK). Capillary electrophoresis was performed on ABI PRISM 3700 DNA Analyzer (POP7) or ABI PRISM 310 Genetic Analyzer (POP6), respectively (both Applied Biosystems, Foster City, CA, USA). Electropherograms were analyzed with SeqScape software version 2.5 (Applied Biosystems).

2.3.11 Mutational analysis of *TP53*

Coding exons 2 to 11 of the *TP53* gene including splice sites were amplified by PCR and subsequently analyzed by direct Sanger sequencing. When a mutation was found, all steps, starting with PCR, were repeated. For detection of possible LGR in the *TP53* gene MLPA was performed at the Institute of Human Genetics, Graz, Austria, as previously described, using the SALSA MLPA kit P056 TP53 (MRC Holland, Amsterdam, the Netherlands) according to the recommendations of the supplier.

2.3.11.1 PCR and direct sequencing

Oligonucleotide primers (see Table 10) and PCR programs (see Table 11) were taken from the protocols of the IARC TP53 Database (July, 2010). Additional primer sets to exon 2 and exon 9 were designed with Primer-BLAST (NCBI) and used in cases where the sequence of the relevant strand was not completely covered during sequencing. An additional primer set (exon8-short) resulting in a shorter PCR product was designed for exon 8 in the case of fragmented DNA. PCR was performed with 50 ng to 100 ng total genomic DNA, 0.2 µM of each primer (Applied Biosystems, Warrington, UK), 200 µM of each dNTP (Applied Biosystems), 1x PCR Buffer (Qiagen, Hilden, DE), which equals 1.5 mM MG^{2+}, and 0.63 to 1.25 U/reaction HotStarTaq DNA Polymerase (Qiagen) in a final volume of 25 µL to 50 µL. A final concentration of 2x Q-Solution (Qiagen) and 3 mM MG^{2+} were used for the primer set exon8-short. A negative control (without template DNA) was included. PCR was performed on the same thermal cyclers as described before (see 2.3.10.1). PCR products were sequenced directly with the same primers already listed and under the same conditions as described for the BRCA1 analysis (see 2.3.10.3).

Table 10. Oligonucleotide primers for *TP53* exon amplification and direct sequencing

Region amplified	Primer pairs (5' to 3')	Product length	PCR program
Exon 2*	CATGGTGTTGGGGGAGGGGGTTC (fw)	274 bp	B
	TCGCTTCCCACAGGTCTCTGCTA (rv)		
Exon 2-3	TCTCATGCTGGATCCCCACT (fw)	344 bp	A
	AGTCAGAGGACCAGGTCCTC (rv)		
Exon 4	TGCTCTTTTCACCCATCTAC (fw)	353 bp	B
	ATACGGCCAGGCATTGAAGT (rv)		
Exon 5	TTCAACTCTGTCTCCTTCCT (fw)	248 bp	B
	CAGCCCTGTCGTCTCTCCAG (rv)		
Exon 6	GCCTCTGATTCCTCACTGAT (fw)	181 bp	B
	TTAACCCCTCCTCCCAGAGA (rv)		
Exon 7	CTTGCCACAGGTCTCCCCAA (fw)	237 bp	C
	AGGGGTCAGCGGCAAGCAGA (rv)		
Exon 8	TTCCTTACTGCCTCTTGCTT (fw)	231 bp	B
	AGGCATAACTGCACCCTTGG (rv)		
Exon 8 (-short*)	TGAGGTGCGTGTTTGTGCCTGT (fw)	88 bp	B
	CAGCTCGTGGTGAGGCTCCC (rv)		
Exon 8-9	TTGGGAGTAGATGGAGCCT (fw)	445 bp	B
	AGTGTTAGACTGGAAACTTT (rv)		
Exon 9*	AGGACAAGAAGCGGTGGAGGAGAC (fw)	284 bp	B
	TCTTTGAGGCATCACTGCCCCCT (rv)		
Exon 10	CAATTGTAACTTGAACCATC (fw)	260 bp	D
	GGATGAGAATGGAATCCTAT (rv)		
Exon 11	AGACCCTCTCACTCATGTGA (fw)	245 bp	B
	TGACGCACACCTATTGCAAG (rv)		

* own primer sets, fw, forward, rv, reverse, bp, base pairs

Table 11. PCR programs for *TP53*

A	Initial denaturation at 95°C for 15 min; 50 cycles of denaturation (94°C for 30 s), annealing (61°C for 45 s), extension (72°C for 45 s); final extension time 10 min
B	Initial denaturation at 95°C for 15 min; 50 cycles of denaturation (94°C for 30 s), annealing (20 cycles 63°C for 45 s, -0.5 °C every 3 cycles; 30 cycles 60°C for 45 s), extension (72°C for 1 min); final extension time 10 min
C	Initial denaturation at 95°C for 15 min; 50 cycles of denaturation (94°C for 30 s), annealing (60°C for 30 s), extension (72°C for 30 s); final extension time 10 min
D	Initial denaturation at 95°C for 15 min; 50 cycles of denaturation (94°C for 30 s), annealing (20 cycles 58.5°C for 45 s, -0.5 °C every 3 cycles; 30 cycles 55°C for 45 s), extension (72°C for 1 min); final extension time 10 min

2.3.11.2 MSP

Cytosine residues were converted using the MethylCode Bisulfite Conversion Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Primers were designed with Methyl Primer Express version 1.0 (Applied Biosystems). The sequence of the primers (5' to 3') was as follows: methylated-specific, 5'-GGAGCGTGTTTTTTACGAC-3' (forward), 5'-ACGATTTTCCCGAACTAAAA-3' (reverse); unmethylated-specific, 5'-TTGGGAGTGTGTTTTTTATGAT-3' (forward), 5'-CCAACAATTTTCCCAAACTAAAA-3' (reverse). The reaction mix consisted of 500 ng total bisulfate treated DNA, 0.15 µM of each primer (Applied Biosystems, Warrington, UK), 200 µM of each dNTP (Applied Biosystems), 1x PCR Buffer (Qiagen, Hilden, DE), additional $MgCl_2$ for a final concentration of 3 mM MG^{2+}, and 0,625 U/reaction HotStarTaq DNA Polymerase (Qiagen) in a final volume of 25 µL. For the reaction with unmethylated-specific primers 1x Q-Solution (Qiagen) was added. CpGenome Universal Methylated as well as Unmethylated (vial A and B) DNA Sets (Millipore, Temecula, CA, USA) were included as positive and negative controls. Furthermore, bisulfate untreated genomic DNA was used to determine whether unspecific amplification occurred. PCR was performed on the same thermal cyclers as described before (see 2.3.10.1) with the following programs: initial denaturation at 95°C for 15 min; 30 cycles of denaturation (94°C for 30 s), annealing (60°C [methylated-specific] or 57°C [unmethylated-specific] for 30 s) and extension (72°C for 1 min); final extension time 10 min.

2.3.12 Mutational analysis of *PTEN*

PCR and direct sequencing were performed under the same conditions as described for the *TP53* gene (see 2.3.11). All nine coding exons as wells as intron-exon boundaries were analyzed. Oligonucleotide primers (see Table 12) were designed with Primer-BLAST (NCBI). MLPA for detection of LGR was conducted with the SALSA MLPA KIT P225-A1 PTEN (MRC Holland, Amsterdam, the Netherlands).

Table 12. Oligonucleotide primers for *PTEN* exon amplification and direct sequencing

Region amplified	Primer pairs (5' to 3')	Product length	PCR program°
Exon 1	AGGGGCATCAGCTACCGCCAAG (fw)	276 bp	B
	ACGGACATTTTCGCATCCGTCTACT (rv)		
Exon 2	ACTGTCCATGTGGAAGTTACCT (fw)	388 bp	D
	TCACAAAGTATCTTTTTCTGTGGCT (rv)		
Exon 3	ACCCATAGAAGGGGTATTTGTTGGA (fw)	367 bp	B
	ACAATGCTCTTGGACTTCTTGACT (rv)		
Exon 4	AGATTCAGGCAATGTTTGTTAGT (fw)	231 bp	D
	TCACTCGATAATCTGGATGACTCA (rv)		
Exon 5	TACCTGTTAAGTTTGTATGCAACAT (fw)	387 bp	D
	TCTCAGATCCAGGAAGAGGAAAGG (rv)		
Exon 6	CCATAGCAATTTAGTGAAATAACTA (fw)	335 bp	D
	GTCTCCTGCATAAATTTCAAATGTG (rv)		
Exon 7	TCCATATTTCGTGTATATTGCTGAT (fw)	391 bp	D
	CACCTGCAGATCTAATAGAAAACA (rv)		
Exon 8	GTTTAACATAGGTGACAGATTTTCT (fw)*	420 bp	D
	TCAAGCAAGTTCTTCATCAGCTG (rv)		
Exon 9	GATGAGTCATATTTGTGGGTTTTCA (fw)	267 bp	D
	TTCATGGTGTTTTATCCCTCTTGA (rv)		

fw, forward; rv, reverse; bp, base pairs; *does not work well for sequencing; °see Table 11

3 RESULTS

3.1 Patients and Primary Diagnoses

Thirty-three (65%) index patients were female and eighteen (35%) were male. Three (6%) index patients were minors, i.e. patients who were under eight-teen years old. The median age at diagnosis of t-MN was 62 (2-85; mean age 56) years. The distribution of the primary diagnoses of the index patients with t-MNs is listed in Table 13. Twenty-five (49%) index patients had primary hematologic malignancy with non-Hodgkin's lymphoma (NHL; 15 patients [29%]) accounting for 60% of all hematologic malignancies. Twenty-six (51%) index patients had a solid tumor as the primary malignancy. Breast cancer was the most common among these (13 patients [25%]) followed by sarcoma (3 patients [6%]).

Table 13. Primary diagnoses of the t-MN patients included in this study

Primary diagnosis	No. patients (%)
Hematologic malignancies	**25 (49)**
Non-Hodgkin's lymphoma	15 (60)
Acute myeloid leukemia	4 (16)
Hodgkin's disease	2 (8)
Myeloma	2 (8)
Acute lymphoblastic leukemia	2 (8)
Solid tumors	**26 (51)**
Breast	13 (50)
Sarcoma	3 (12)
Colon	2 (8)
PNET, Ewing´s sarcoma	2 (8)
Ovary	1
Uterus	1
Thyroid gland	1
Prostate	1
Germ cell	1
Hepatic hemangiomatosis	1

PNET, Primitive Neuroectodermal Tumor

Of the 25 index patients with hematologic malignancies eleven (44%) patients received only chemotherapy (CT), one (4%) patient was treated with radiation therapy (RT) only and thirteen (52%) patients had combined modality treatment (CMT) for their primary malignancy, either simultaneously or consecutively. The corresponding numbers for the 26 index patients with solid tumors are seven (CT [27%]), ten (RT [38%]) and nine (CMT [35%]), respectively (Table 14).

Table 14. Primary diagnoses and respective therapy modalities

Primary diagnosis	No. Patients	CT only (%)	RT only (%)	CMT (%)
Hematologic malignancy	25	11 (44)	1 (4)	13 (52)
Solid tumor	26	7 (27)	10 (38)	9 (35)

CT, chemotherapy; RT, radiation therapy; CMT, combined modality therapy

Twenty-three (45%) patients presented with t-MDS, 26 (51%) patients with t-AML and two (4%) patients with t-MDS/MPN, respectively. Fourteen patients (61%) had progression from t-MDS to t-AML at a median time of three months. The median latency interval from cytotoxic therapy to development of t-MN was 53 months (Table 15).

Table 15. Clinical presentation of the t-MN patients

No. Patients	t-MDS (%)	t-AML (%)	t-MDS/ MPN (%)	t-MDS→t-AML (%)	Median latency in months
51	23 (45)	26 (51)	2 (4)	14 (61)	53

Data of cytogenetic analysis of t-MNs was available in 48 index patients (94%) and is listed in Table 16. Only eight patients (17%) had a normal karyotype, whereas 17 patients (38%) had unfavorable complex karyotypes. Aberrations involving chromosomes 7 and 5 which are associated with t-MNs were the most common.

Table 16. Bone marrow cytogenetic analysis of the t-MN patients

Cytogenetic status	No. (%)
Available	48 (94)
Normal karyotype	8 (17)
Clonal aberrations	40 (83)
Complex karyotype	16 (38)
-7, del(7q)	18 (38)
-5, del(5q)	15 (31)
11q23 rearrangements	6 (13)
Chromosome 3 abnormalities	6 (13)
inv(16)	4 (8)

3.2 Characteristics of Index Patients tested for Germ-line Mutations

Characteristics of index patients tested for germ-line mutations are described in Table 17. The evaluation of the fifty-one pedigrees showed no clear evidence of known hereditary cancer syndromes in the group of patients with hematologic disease as primary malignancy. By contrast, index patients with solid tumor as primary malignancy showed familiar clustering of tumors, which was especially apparent in patients treated for breast cancer. Index patients indicating hereditary cancer predisposition were subdivided into the predefined groups A, B and C (see 2.1.2).

Table 17. Characteristics of index patients tested for germ-line mutations (continued on the next page)

Group	UPN	Primary tumor/s (age*)	Primary therapy°	t-MN (age*), latency of transformation	Cytogenetic and molecular aberrations of t-MN	Age of death*
A	6732	**Osteosarcoma** (29), capillary hemangioma (30)	CT (AA, AM, TI)	t-AML (31)	*MLL* rearrangement	31
	6755	**Ewing´s sarcoma** (22), malignant melanoma (23)	CMT (AA, TI)	t-AML (27)	del(7)(q11q36), t(11;17)(q23;q25); *MLL* rearrangement	alive

Group	UPN	Primary tumor/s (age*)	Primary therapy°	t-MN (age*), latency of transformation	Cytogenetic and molecular aberrations of t-MN	Age of death*
B	5294	CIN III (28), **Breast cancer** (left 34, right 39)	CMT (AT, TI)	t-AML (47)	trisomy X, -22	47
	5824	**Breast cancer** (left 27, right 32)	CMT (AT, TI)	t-MDS> t-AML# (35)	del(7)	36
	5869	Ovarian cancer (56)	CT (AT, TI)	t-MDS> t-AML (61), 6 months	5q-	61
	6353	Breast cancer (57)	CMT (AA, TI)	t-MDS> t-AML# (62)	CK, del(5q)	63
	6387	Breast cancer (60)	RT	t-AML (65)	+8	alive
	7029	Breast cancer (53)	RT	t-AML (68)	t(16;16)(p13;q22)	alive
C	5129	Colorectal adenoma (35), uterine fibroids (48),**breast cancer** (48)	RT	t-AML (50)	*MLL* rearrangement	51
	6144	uterine myoma (58), **Breast cancer** (67), renal cancer (78), borderline ovarian tumor (78)	CT (AA, TI)	t-AML (79)	not performed	80
	6371	**Breast cancer** (50), angiomyolipoma (r), enchondroma (r)	RT	t-MDS> t-AML# (54)	CK, del(3), del(5), del(11)	alive
	6925	Thyroid cancer (37)	RIT	biphenotypic t-AL (48)	CK, del(5q), del(11q), del(7), del(17)	52

*years; #direct transformation;°according to Smith *et al*. (Smith et al. 2003b); bold characters indicate the malignancy patients got cytotoxic treatment for

AA, alkylating agents; AL, acute leukemia; AM, antimetabolites; AT, antitubulin agents; CA, complex aberrations; chr., chromosome; CIN, cervical intraepithelial neoplasia; CT, chemotherapy; CMT, combined modality treatment; *MML*, mixed lineage leukemia gene; r, radiodiagnosis; RIT, radioactive Iodine therapy; RT, radiation therapy; TI, topoisomerase 2 inhibitors; u, age unknown; UPN, unique patient number

In t-MN patients meeting the criteria of group A, constitutional DNA was available in two index patients (UPN 6732, 6755). Group A included a male index patient (UPN 6732) with osteosarcoma who met the original and the revised Chompret criteria for LFS/LFL (Chompret et al. 2001, Tinat et al. 2009), and a female index patient (UPN 6755) who met the criteria for LFL proposed by Eeles (Eeles 1995). The latter had a sarcoma which could not be distinguished into small cell osteosarcoma or Ewing´s sarcoma by microscopic examination. Molecular diagnostics showed a EWS-FLI-1 fusion protein which made the more probable diagnosis of Ewing´s sarcoma (Ginsberg et al. 1999).

Six patients fulfilled the S3 criteria for hereditary breast and ovarian cancer syndrome (group B) (Kreienberg et al. 2008). In five of these patients (UPN 5294, 5824, 5869, 6353, 6387) constitutional DNA from fibroblasts was available. One patient refused punch biopsy so that DNA from Ficoll separated leucocytes (UPN 7029) obtained in a state of complete remission of the t-AML was used for testing.

Four patients (UPN 5129, 6144, 6371, 6925) fulfilled the preconditions of group C. They were tested first for *TP53* (LFL) and subsequently for *PTEN* (Cowden syndrome). One index patient (UPN 6925) with thyroid cancer showed a marked family history for colorectal carcinoma and breast cancer, and had been tested previously with negative results for Lynch syndrome (microsatellite instability) and *MUTYH*-associated polyposis (group C). Mutational analysis of candidate genes reveals clinically relevant variations in *BRCA1*, *TP53* and *BARD1*

Mutational analyses were performed on genomic DNA derived from fibroblasts, frozen skin tissue (UPN 6732) and from leukocytes (UPN 7029). The results with all genetic variations of *BRCA1*, *BRCA2* and *TP53* are shown in Table 18, in Table 19 and in Table 20, respectively.

Table 18. Results of *BRCA1* testing

UPN	Exon	Nt change	AA change	RefSNP ID
5294	10-exon	c.2082C>T, het.	-	rs1799949
	10-exon	c.2311T>C, het.	-	rs16940
	10-exon	c.2612C>T, het.	p.P871L	rs799917
	10-exon	c.3113A>G, het.	p.L1038P	rs16941
	10-exon	c.3548A>G, het.	p.F1183S	rs16942
	12-exon	c.4308T>C, het.	-	rs1060915
	15-exon	c.4837A>G, het.	p.S1613G	rs1799966
5824	**20-exon**	**c.5251C>T, het.**	**p.R1751***	**rs80357123**
5869	**10-exon**	**c.3112G>T, het.**	**p.E1038***	**rs80357161**
	10-exon	c.3119G>A, het.	p.S1040N	rs4986852
6353	-	no sequence variations	-	-
6387	10-exon	c.2082C>T, het.	-	rs1799949
	10-exon	c.2311T>C, het.	-	rs16940
	10-exon	c.2612C>T, het.	p.P871L	rs799917
	10-exon	c.3113A>G, het.	p.L1038P	rs1694
	10-exon	c.3548A>G, het.	p.F1183S	rs169421
	12-exon	c.4308T>C, het.	-	rs1060915
	15-exon	c.4837A>G, het.	p.S1613G	rs1799966
7029	10-exon	c.2082C>T, hom.	-	rs1799949
	10-exon	c.2077G>A, het.	p.D639N	rs4986850
	10-exon	c.2311T>C, hom.	-	rs16940
	10-exon	c.2612C>T, hom.	p.P871L	rs799917
	10-exon	c.3113A>G, hom.	p.L1038P	rs16941
	10-exon	c.3548A>G, hom.	p.F1183S	rs16942
	12-exon	c.4308T>C, hom.	-	rs1060915

AA, amino acid; het., heterozygous; hom., homozygous; Nt, nucleotide; UPN, unique patient number; bold characters indicate deleterious or clinically associated variations

UCSC, assembly February, 2009 (GRCh.37/hg19), dbSNP build 131

Table 19. Results of *BRCA2* testing

UPN	Exon	Nt change	AA change	RefSNP ID
5294	10-exon	c.1114A>C, hom.	p.N327H	rs144848
	11-exon	c.4563A>G, hom.	-	rs206075
	11-exon	c.6513G>C, hom.	-	rs206076
	14-exon	c.7397T>C, hom.	p.V2466A	rs169547
5824	10-exon	c.1114A>C, het.	p.N372H	rs144848
	11-exon	c.3396A>G, het.	-	rs1801406
	11-exon	c.4563A>G, hom.	-	rs206075
	11-exon	c.6513G>C, hom.	-	rs206076
	14-exon	c.7242A>G, het.	-	rs1799955
	14-exon	c.7397T>C, hom.	p.V2466A	rs169547
5869	11-exon	c.3396A>G, hom.	-	rs1801406
	11-exon	c.4563A>G, hom.	-	rs206075
	11-exon	c.6513G>C, hom.	-	rs206076
	14-exon	c.7242A>G, het.	-	rs1799955
	14-exon	c.7397T>C, hom.	p.V2466A	rs169547
6353	11-exon	c.3807T>C, het.	-	rs543304
	11-exon	c.4563A>G, hom.	-	rs206075
	11-exon	c.6513G>C, hom.	-	rs206076
	14-exon	c.7397T>C, hom.	p.V2466A	rs169547
6387	10-exon	c.865A>C, het.	p.N289H	rs766173
	10-exon	c.1365A>G, het.	-	rs1801439
	11-exon	c.2229T>C, het.	-	rs1801499
	11-exon	c.2971A>G, het.	p.N991D	rs1799944
	11-exon	c. 4563A>G, hom.	-	rs206075
	11-exon	c.6513G>C, hom.	-	rs206076
	14-exon	c.7397T>C, hom.	p.V2466A	rs169547
7029	11-exon	c.3396A>G, het.	-	rs1801406
	11-exon	c.3807T>C, het.	-	rs543304
	11-exon	c.4563A>G, hom.	-	rs206075
	11-exon	c.6513G>C, hom.	-	rs206076
	14-exon	c.7242A>G, het.	-	rs1799955
	14-exon	c.7397T>C, hom.	p.V2466A	rs169547

AA, amino acid; het., heterozygous; hom., homozygous; Nt, nucleotide; UPN, unique patient number

UCSC, assembly February, 2009 (GRCh.37/hg19), dbSNP build 131

Table 20. Results of *TP53* testing

UPN	Exon	Nt change	AA change	RefSNP ID
5129	4-exon	c.215C>G, hom.	p.P72R	rs1042522
5294	4-exon	c.215C>G, het.	p.P72R	rs1042522
6144	4-exon	c.215C>G, hom.	p.P72R	rs1042522
	11-exon	**c.1146delA, het.**	**p.K382fs*40**	**-**
6353	4-exon	c.215C>G, het.	p.P72R	rs1042522
6371	4-exon	c.215C>G het.	p.P72R	rs1042522
	8-exon	**c.849_852insGGCG, het.**	**p.R283fs*22**	**-**
6387	4-exon	c.215C>G, het.	p.P72R	rs1042522
6732	-	no sequence variations	-	-
6755	4-exon	c.215C>G, hom.	p.P72R	rs1042522
6925	4-exon	c.215C>G, hom.	p.P72R	rs1042522
7029	4-exon	c.215C>G, het.	p.P72R	rs1042522

AA, amino acid; het., heterozygous; hom., homozygous; Nt, nucleotide; UPN, unique patient number; bold characters indicate deleterious variations

NM_000546.4 (*TP53*); dbSNP build 131

Among the twelve index patients tested for germ-line mutations no large genomic rearrangements could be found by MLPA. Unfortunately, due to low yield of DNA purification MLPA analysis of *TP53* was not possible in index patient UPN 6732, whose DNA was extracted directly from frozen skin tissue.

No *TP53* variations were found in patients of group A who fulfilled clinical criteria for *TP53* germ-line mutation testing.

Sequencing revealed multiple common polymorphisms at various positions in the *BRCA* genes in index patients of group B. Three synonymous and six non-synonymous variations were found in *BRCA1* and seven synonymous and four non-synonymous variations in *BRCA2*, respectively. Additionally, two index patients had heterozygous

nonsense mutations in the *BRCA1* gene, and one patient had a heterozygous non-synonymous variation in *BARD1*.

The c.5251C>T transition (UPN 5824) is a known deleterious *BRCA1* nonsense mutation with multiple entries in the Breast Cancer Core Database (BIC, research.nhgri.nih.gov/bic) leading to a premature stop codon in exon 20 of *BRCA1*. The second nonsense mutation, the c.3112G>T transversion (UPN 5869), terminates translation at position 1038 in exon 10 of *BRCA1*. As of March 2011, c.3112G>T has been reported only once in the BIC database and classified as clinically important.

The c.1670G>C (UPN 6387) non-synonymous variation in the exon 7 of *BARD1* (see Figure 6) leads to a cysteine to serine residue substitution at position 557. This variation is reported with a minor allele frequency of less than 3% in dbSNP (rs28997576; www.ncbi.nlm.nih.gov/projects/SNP). The C557S variation has probable clinical relevance since its frequency was observed to be higher in women with family history of breast cancer (Karppinen et al. 2004). Furthermore, breast cancer cells transfected with the C557S allele had reduced growth suppression and apoptosis capabilities (Sauer, Andrulis 2005).

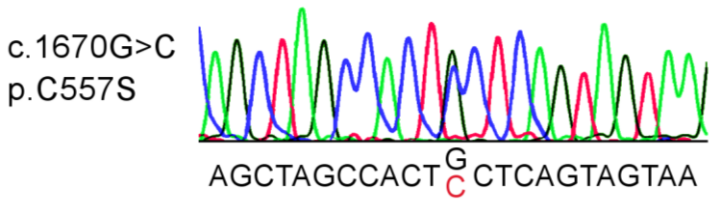

Figure 6. Heterozygous *BARD1* c.1670G>C germ-line mutation found in fibroblasts of index patient UPN 6387

Index patients in group B who had no deleterious mutations in the *BRCA* genes did not show deleterious mutations in *TP53* and *PTEN,* either. The remaining four index patients (UPN 5294, 6353, 6387, 7029) of group B without deleterious *BRCA1/2* mutations had heterozygous c.215C>G variation located in exon 4 of *TP53,* which is a validated polymorphism with an average frequency of heterozygotes of 0.49 according to the IARC TP53 Database (www-p53.iarc.fr). The c.215C>G transversion in codon 72 results in a proline to arginine (P72R) substitution.

Mutational analysis of the four index patients in group C revealed the TP53 P72R polymorphism in two cases (UPN 6371, 6925). One (UPN 6925) of them was homozygous for the Arginine allele. Futhermore, two deleterious indel mutations in *TP53* were found.

The heterozygous c.1146delA mutation (UPN 6144) in exon 11 of *TP53* generates a frameshift of 40 codons. Translation of the mutated mRNA transcript would result in a potentially 27 amino acids longer p53 protein and loss of the acetylation site at lysine 382 as well as of the phosphorylation site at serine 392, if no nonsense mediated mRNA decay (NMD) occurred. In order that NMD can proceed one intron 5' of a termination codon must be present (Gardner 2010). Since exon 11 is the last coding exon of *TP53*, elongation of p53 in the case of c.1146delA is easily conceivable. The *Catalogue of Somatic Mutations in Cancer* (COSMIC, www.sanger.ac.uk/genetics/CGP/cosmic) lists only one entry for c.1146delA, observed as somatic mutation in a gastric cancer cell line. Moreover, the heterozygous *TP53* c.1146delA mutation was detected in DNA derived from EBV-transformed lymphoblasts in a study of survivors of Hodgkin's disease with second primary malignancy, but could not be verified as germ-line mutation (Nichols et al. 2003). Hence, the occurrence of somatic c.1146delA *TP53* mutation in malignant cells supports the conclusion that c.1146delA is a deleterious mutation. The germ-line nature of c.1146delA was confirmed by analysis of DNA from granulocytes and breast cancer cells, both showing a heterozygous state of mutation.

The second indel mutation, the heterozygous c.849_852insGGCG (UPN 6371) mutation in exon 8 of *TP53*, produces a frameshift of 22 codons resulting in a truncated p53 protein by NMD. The c.849_852insGGCG has not been previously described, neither as somatic nor as germ-line mutation. Confirmation of the germ-line nature was brought by mutational analysis of DNA from whole blood specimen and breast cancer cells showing a heterozygous state once more.

A summary of all clinically relevant germ-line mutations found in this study is given in Table 21.

Table 21. Summary of clinically relevant germ-line mutations found in this study

UPN	Gene	Mutation	Predicted effect	Previous description	Pedigree analysis
5824	*BRCA1*	c.5251C>T p.R1751* rs80357123	- Truncated BRCA1 (NMD) - Clinically important (BIC Database[1])	Multiple entries in BIC Database[1] (29 as of July 2011)	HBOCS
5869	*BRCA1*	c.3112G>T p.E1038* rs80357161	- Truncated BRCA1 (NMD) - Clinically important (BIC Database[1])	One entry in BIC Database[1] (as of July 2011)	HBOCS
6371	*TP53*	c.849-852insGGCG p.R283fs*22	- Truncated p53 (NMD) - Predicted disease causing[2]	none	- Multiple diverse cancers - Probably *de novo* mutation
6144	*TP53*	c.1146delA p.K382fs*40	- Prolonged p53 (+27 AA) - Predicted disease causing[2]	Somatic mutation in a gastric cancer cell line (COSMIC Database[3]) and in EBV-transformed lymphoblasts (Nichols et al. 2003)	- Inconspicuous - Probably *de novo* mutation
6387	*BARD1*	c.1670G>C p.C557S rs28997576	Reduced capability of growth suppression and apoptosis	(Karppinen et al. 2004) (Sauer, Andrulis 2005)	HBOCS

[1]research.nhgri.nih.gov/bic/; [2]mutationtaster.org; [3]sanger.ac.uk/genetics/CGP/cosmic; HBOCS, hereditary breast and ovarian cancer syndrome; NMD, nonsense mediated mRNA decay; UPN, unique patient number

3.3 Pedigrees of Index Patients with Identified Germ-line Mutations

3.3.1 Pedigree UPN 5824

The pedigree of index patient UPN 5824 (III:10) who carried the deleterious *BRCA1* nonsense germ-line mutation c.5251C>T shows classical HBOCS with multiple breast cancer cases and one case of ovarian cancer occurring through three generations (see Figure 7).

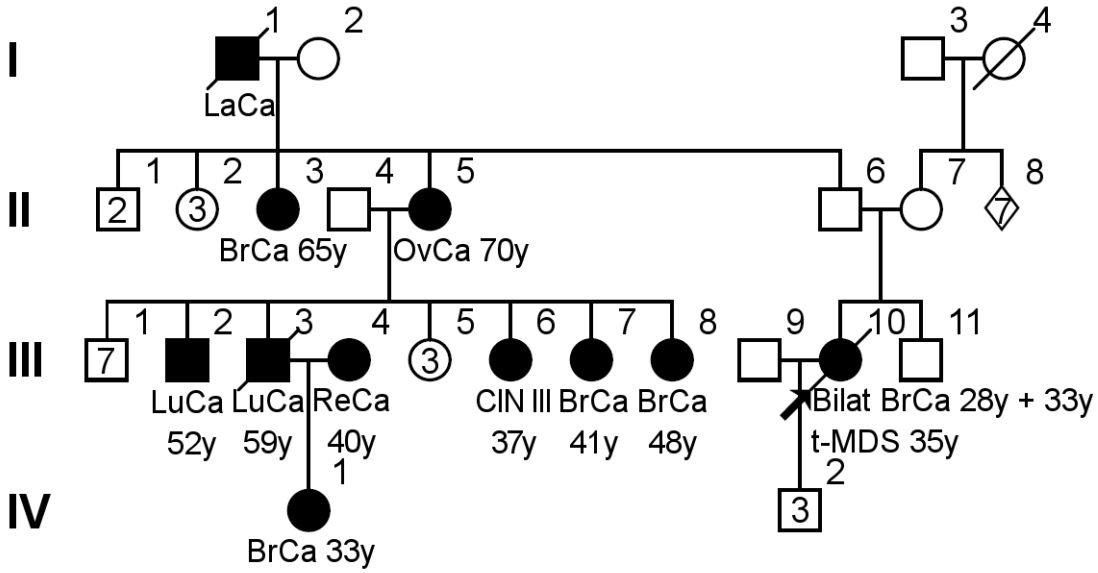

Figure 7. Pedigree of index patient UPN 5824 (arrow). Numbers within symbols indicate the number of unaffected individuals; Bilat, bilateral; BrCa, breast cancer; CIN, cervical intraepithelial neoplasia; LaCa, laryngeal cancer; LuCa, lung cancer; ReCa, renal cancer; y, age in years.

This pedigree fulfilled multiple criteria for *BRCA* gene testing (see Table 8) but was not needed to finally make the supposition of HBOCS since the index patient had early onset bilateral breast cancer at the age of 27 and 32 years, respectively. Interestingly, treating oncologists did not see the necessity to inform the patient about the option of genetic counseling, possibly because breast and ovarian cancers were lacking in first-degree relatives. Genetic analyses revealed that the c.5251C>T *BRCA1* germ-line mutation was inherited from the father's side who himself did not develop cancer. This observation is in accordance with a previously reported study by King at al. which could show that especially patients with inherited *BRCA* mutations and low-incidence family history for breast or ovarian cancer inherited the mutation from their fathers and are missed since they do not fit in the frame (King et al. 2003). Admittedly, this pedigree would not be a

true low-incidence family, if family history was expanded on second- and third-degree relatives. Index patient UPN 5824 was treated with operation, radiation therapy and multiple cycles of epirubicin and docetaxel. At the age of 35 years, t-MDS with monosomy 7 was diagnosed. She succumbed in the course of transformation to t-AML.

3.3.2 Pedigree UPN 5869

The pedigree of index patient UPN 5869 (III:2) with deleterious *BRCA1* nonsense germ-line mutation c.3112G>T demonstrates multiple breast cancers in third degree relatives and gastric cancers in first and second degree relatives (see Figure 8). The index patient UPN 5869 developed ovarian cancer at the age of fifty-six years and was treated with surgery and adjuvant chemotherapy composed of carboplatin and docetaxel. t-MDS with 5q- was diagnosed at the age of 61. She finally died of t-AML transformed from t-MDS within six months.

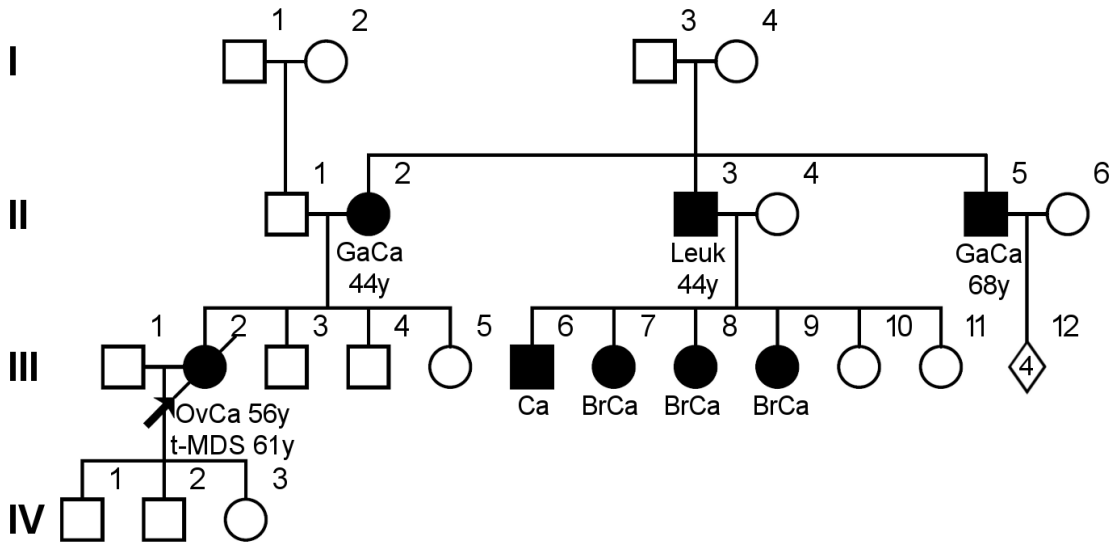

Figure 8. Pedigree of index patient UPN 5869 (arrow). Numbers within symbols indicate the number of unaffected individuals; BrCa, breast cancer; GaCa, gastric cancer; Ca, cancer of unknown origin; Leuk, leukemia of unknown type; OvCa, ovarian cancer; y, age in years.

It has been postulated that *BRCA1* germ-line mutations increase the risk for development of gastric cancer especially in men (Mohamad, Apffelstaedt 2008). Since no segregation analysis was performed, a final statement regarding the route of inheritance cannot be done. Nevertheless, it is highly likely that *BRCA1* germ-line mutation was inherited from the mother's side which shows multiple breast cancer cases in third-degree relatives (III:7-9) by family history. If this assumption was right, then the

occurrence of two gastric cancers in two uncles would be conspicuous. It should be of note that all declared cancers were not verified by medical records.

3.3.3 Pedigree UPN 6387

Index patient UPN 6387 (III:2) was tested negative for mutations in the *BRCA* genes but was found having c.1670G>C non-synonymous variation in *BARD1*. She suffered from breast cancer at the age of sixty years and was treated with surgery, radiation therapy and adjuvant tamoxifen. Sixty months later, t-AML with trisomy 8 was diagnosed. Her pedigree shows ovarian cancer in first-degree and two breast cancer cases in third-degree relatives, hence indicating a classical HBOCS (see Figure 9). Cancer cases are unverified and were only found out by family history. No segregation analysis was performed.

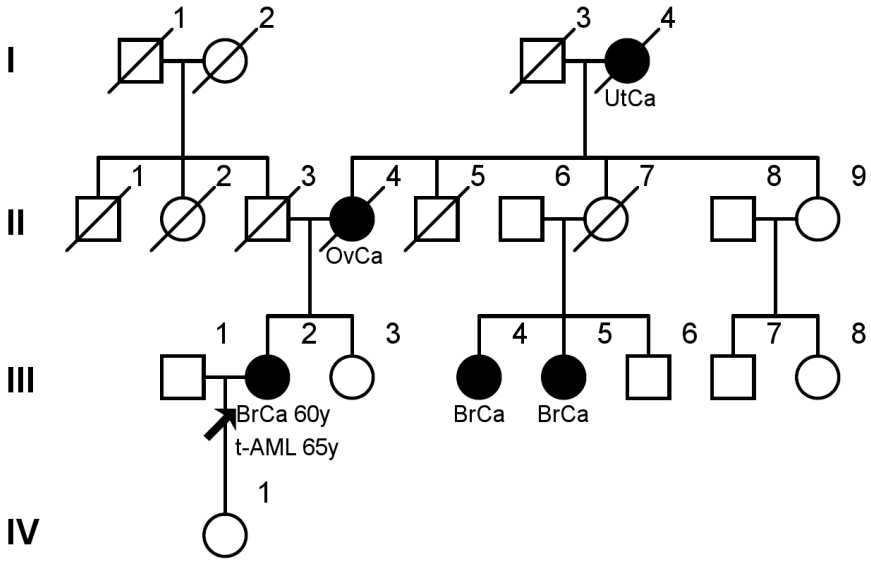

Figure 9. Pedigree of index patient UPN 6387 (arrow). BrCa, breast cancer; OvCa, ovarian cancer; UtCa, uterine cancer; y, age in years.

3.3.4 Pedigree UPN 6371

Index patient UPN 6371 (III:2) having heterozygous c.849_852insGGCG TP53 germ-line mutation developed breast cancer at the age of 50 years and was treated with surgery and radiation therapy. Additionally, two benign neoplasms, enchondroma and angiomyolipoma, were diagnosed by radiology examinations, but received no further therapy. At the age of fifty-four years, t-MDS with a complex karyotype including deletions of chromosomes 3, 5 and 11 occurred. She received successfully allogeneic non-

myeloablative stem cell transplantation during partial remission. Her pedigree shows multiple, diverse malignancies in first and second degree relatives (see Figure 10).

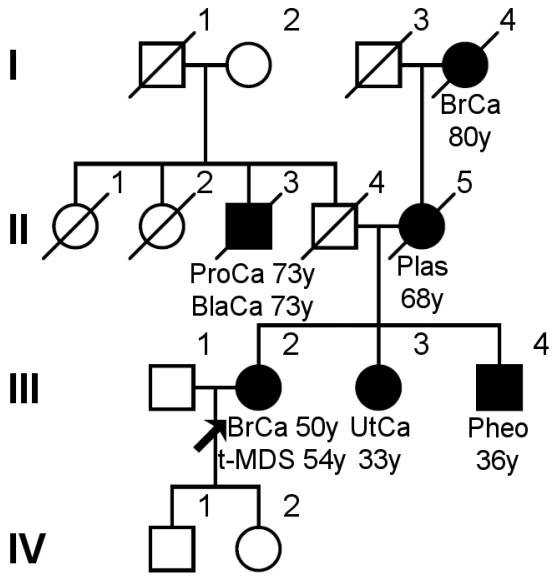

Figure 10. Pedigree of index patient UPN 6371 (arrow). BlaCa, bladder cancer; BrCa, breast cancer; Pheo; pheochromocytoma; Plas, plasmacytoma; ProCa, prostate cancer; UtCa, uterine cancer; y, age in years.

Conformation of segregation was sought in the index patient's family. Both siblings (III:3-4) and both children(IV:1-2) as well as one affected uncle (II:3) were tested negative for the c.849-852insGGCG germ-line mutation. Due to DNA fragmentation PCR of DNA extracted from the mother's plasmacytoma was not feasible. Thus, it is still unknown whether this *TP53* germ-line mutation was inherited or developed *de novo*.

3.3.5 Pedigree UPN 6144

Patient UPN 6144 suffered from multiple primary neoplasms including breast and renal cancer at the age of 68 and 78 years, respectively. She received four cycles of epirubicin and cyclophosphamide as adjuvant therapy after mastectomy and axillary dissection, whereas renal cancer was treated with nephrectomy only. She developed t-AML at the age of 79 and succumbed to it shortly after. Unfortunately, karyotype is unknown because no cytogenetic analysis of the t-AML has been performed. Pedigree analysis did not show any relatives affected with cancer (not shown). No segregation analysis was performed.

3.4 Analysis of Deleterious Germ-line Mutations in Leukemic Cells

DNA extracted from isolated CD34$^+$ leukemic cells of affected index patients obtained at t-MN diagnosis was analyzed to assess somatic zygosity of the encountered deleterious germ-line mutations in *BRCA1* and *TP53*. For this purpose PCR and direct sequencing were performed. In cases of homozygosity of the mutant allele, analysis was amended with SNP array to assess the mode of inactivation of the wild-type allele. Due to lack of appropriate leukemia samples from index patient UPN 6387, CD34$^+$ leukemic cells could not be analyzed for the *BARD1* C557S mutation.

3.4.1 Direct sequencing shows homozygosity in CD34$^+$ cells for two deleterious germ-line mutations

Direct sequencing of DNA from isolated CD34$^+$ leukemic cells revealed homozygosity of c.3112G>T in *BRCA1* (see Figure 11) and c.849_852insGGCG in *TP53* (see Figure 12), respectively.

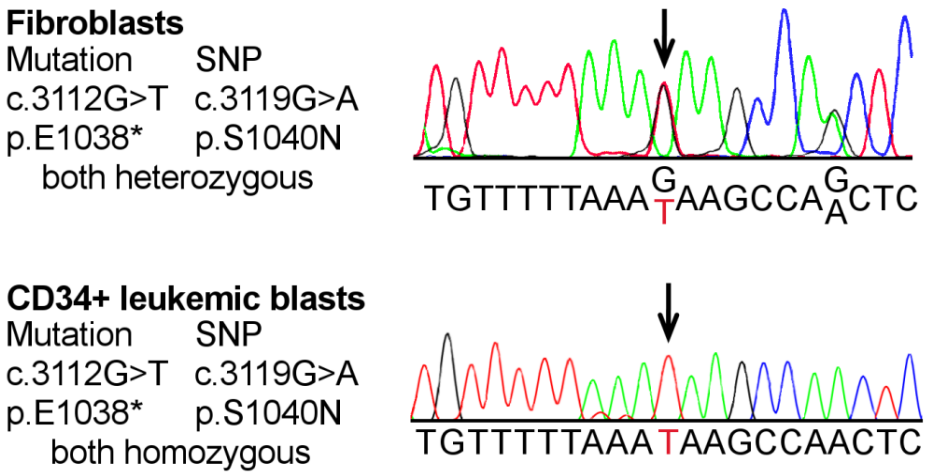

Figure 11. *BRCA1* c.3112G>T mutation (arrow) in fibroblasts and CD34$^+$ leukemic cells of index patient UPN 5869.

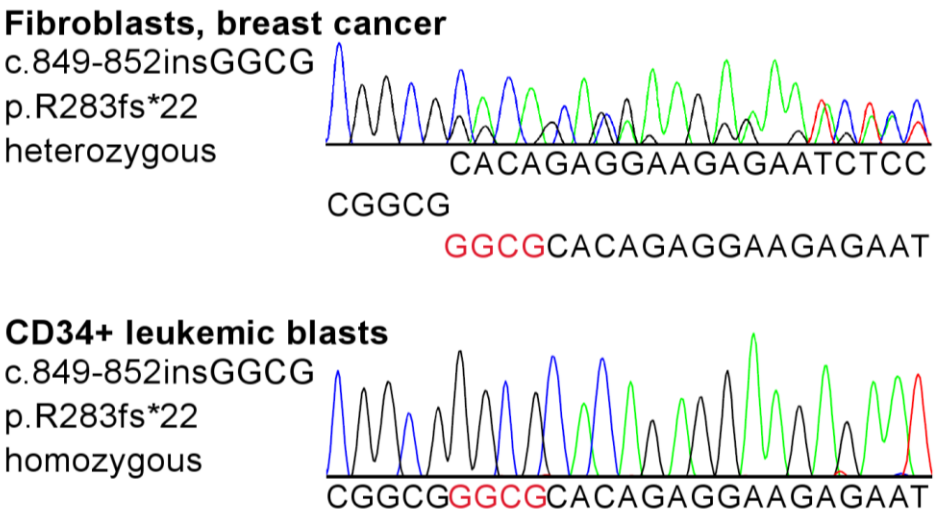

Figure 12. *TP53* c.849-852insGGCG mutation in fibroblasts and CD34$^+$ leukemic cells of index patient UPN 6371.

The nonsense mutation c.5251C>T in *BRCA1* and the deletion c.1146delA in *TP53* remained both heterozygous in leukemic blasts (see Figure 13 and Figure 14, respectively).

In cells with a heterozygous mutation of a certain gene, the wild-type and mutated allele can be expressed differently. To determine whether wild-type alleles were silenced in CD34$^+$ leukemic cells, cDNA was analyzed. Unfortunately, only mRNA from the leukemic cells of index patient UPN 5824 with c.5251C>T *BRCA1* mutation could be obtained. Direct sequencing of reverse-transcribed cDNA revealed heterozygous c.5251C>T (see Figure 13). Hence, on a qualitative level, both copies of *BRCA1* were transcribed to mRNA.

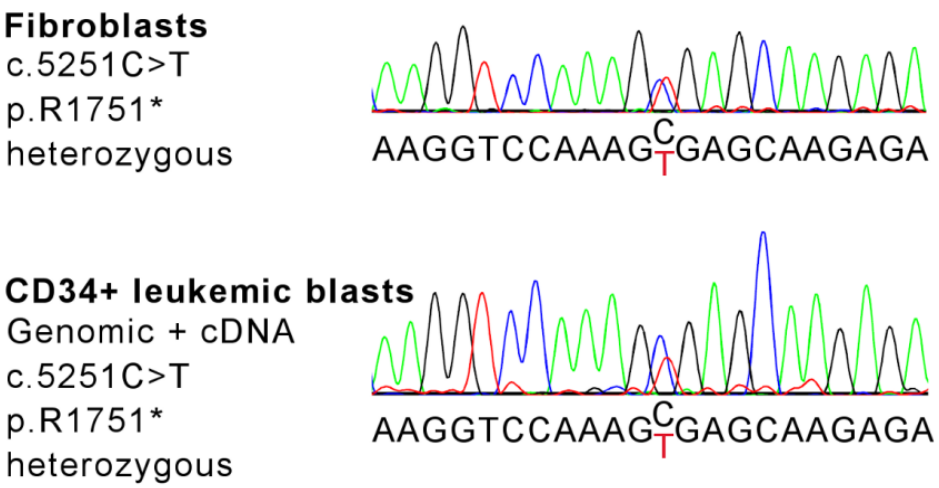

Figure 13. ***BRCA1*** **c.5251C>T mutation in fibroblasts and CD34$^+$ leukemic cells of index patient UPN 5824.**

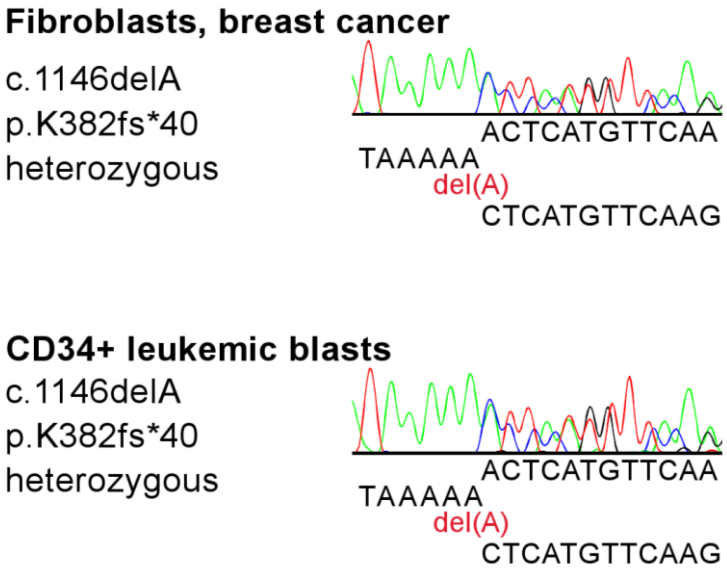

Figure 14. ***TP53*** **c.1146delA mutation in fibroblasts and CD34$^+$ leukemic cells of index patient UPN 6144.**

3.4.2 SNP array identifies loss of heterozygosity of the wild-type allele

Virtual karyotypes of leukemic cells of index patients UPN 5869 and UPN 6371 are depicted in Figure 15. In addition to the known 5q- aberration, deletions of chromosomes 6, 8, 17 and 18 were demonstrated in index patient UPN 5869. Similarly, virtual karyotyping added broad deleted regions in chromosomes 12, 13, 15 and 17 in addition to the known deletions in chromosomes 3, 5 and 11 identified by conventional metaphase cytogenetic studies in UPN 6371.

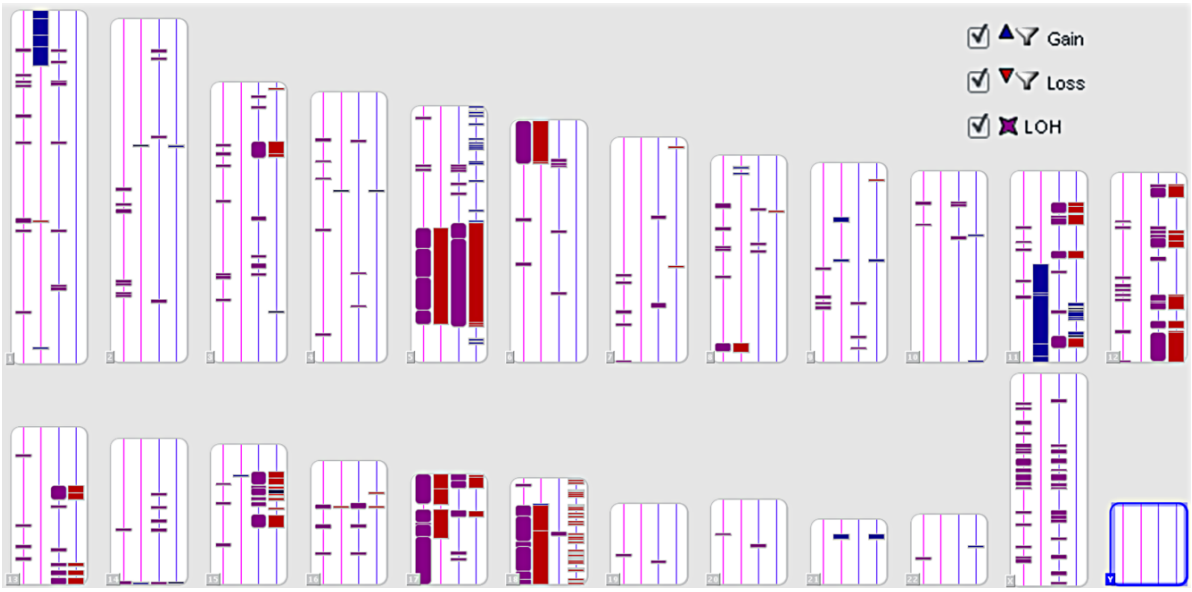

Figure 15. Virtual karyotypes of leukemic cells of index patients UPN 5869 (left two lines) and UPN 6371 (right two lines) show multiple copy number variations. Blue regions indicate gains, red regions losses and violet regions LOH.

For both genes, *BRCA1* in index patient UPN 5869 and *TP53* in UPN 6371, SNP array demonstrated deletions of respective loci and copy number states equaling 1 (see Figure 16 and Figure 17). Hence, in both cases classical LOH with deletion of the wild-type allele occurred.

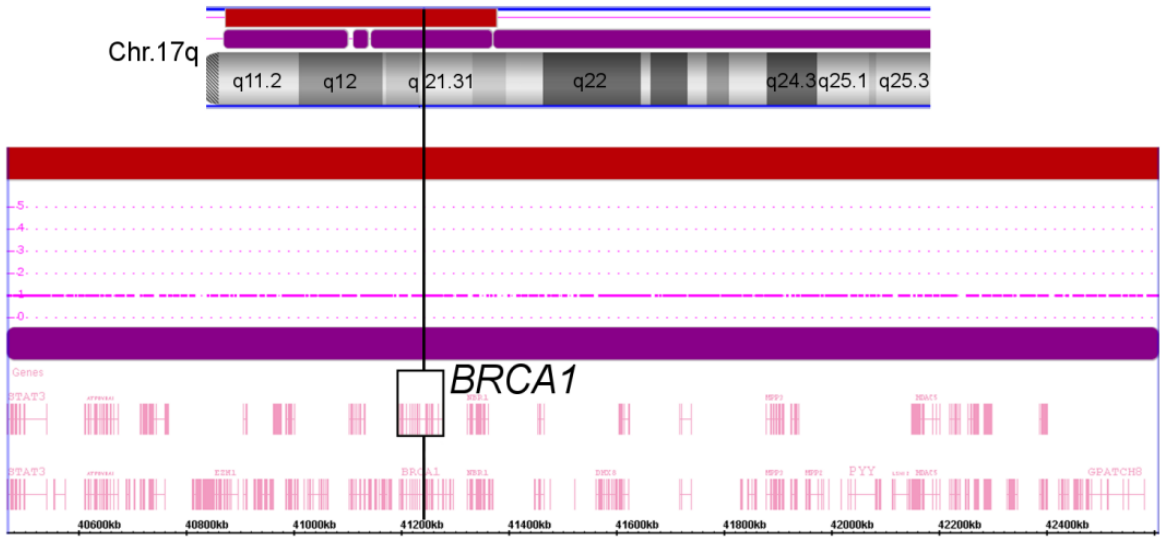

Figure 16. *BRCA1* locus on chromosome 17q of leukemic cells of index patient UPN 5869 shows copy number state 1 and LOH. Red regions indicate losses and violet regions LOH.

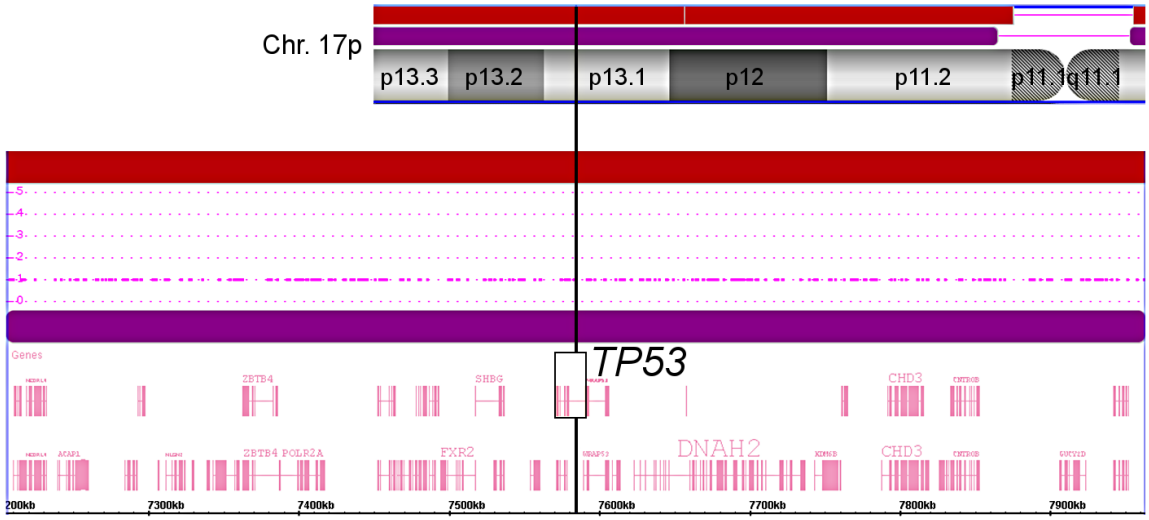

Figure 17. *TP53* locus on chromosome 17p of leukemic cells of index patient UPN 6371 shows copy number state 1 and LOH. Red regions indicate losses and violet regions LOH.

3.5 Hypermethylation of the Promoter is No Mechanism of Inactivation of the Wild-type Allele in Both *TP53* Germ-line Mutations

Since mRNA of $CD34^+$ leukemic blasts from index patient UPN 6144 as wells as mRNA of breast cancer tissue from index patients 6371 could not be analyzed for differential transcription of *TP53* due to unavailability of tissue samples, indirect proof was sought in genomic DNA isolated from aforementioned tissues.

Methylation of promoters is a frequent mechanism of tumor suppressor silencing in human cancers (Herman et al. 1996). To determine the methylation status of the *TP53* promoter, MSP was performed with DNA from the breast cancer tumor as well as from leukemic cells of index patient UPN 6144 and from breast cancer tissue of index patient UPN 6371, respectively. DNA from fibroblasts was used as constitutional control in both cases. MSP showed no hypermethylation of the *TP53* promoter in $CD34^+$ leukemic cells, breast cancer tumor and fibroblasts of index patient UPN 6144 (see Figure 18), respectively. Likewise, no hypermethylation could be seen in the breast cancer tumor of index patient UPN 6371 (see Figure 19).

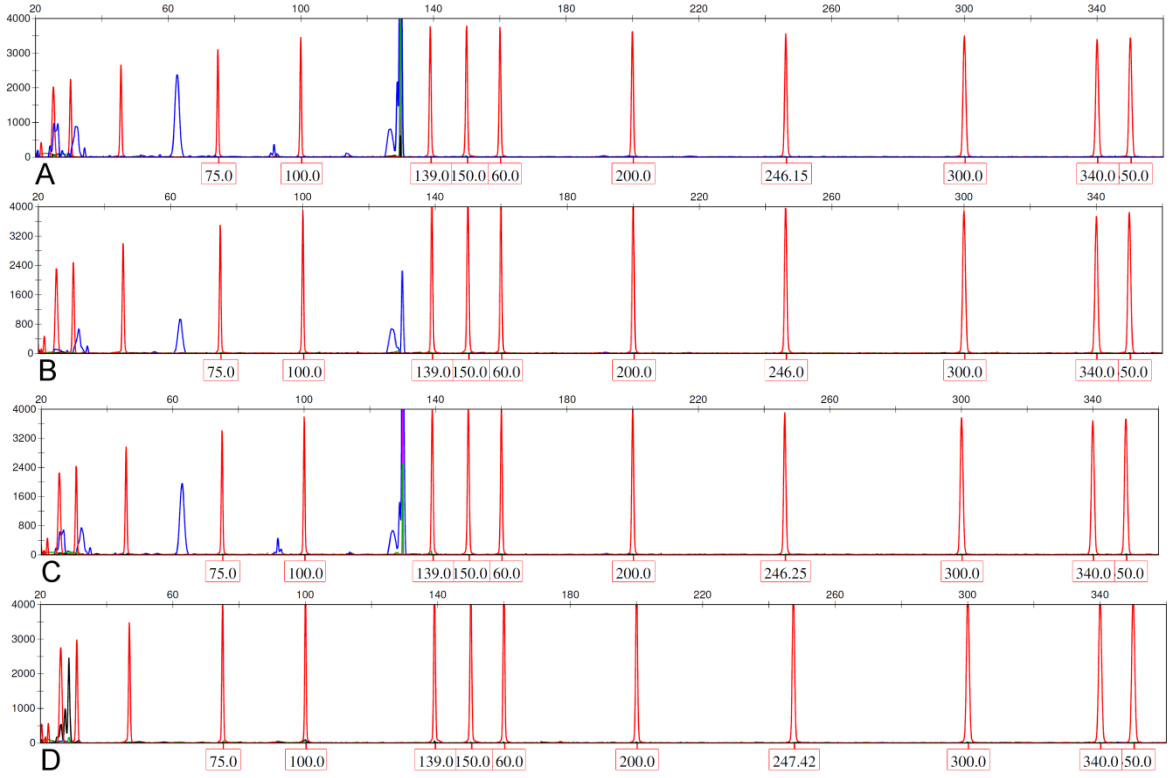

Figure 18. MSP of genomic DNA from CD34+ leukemic cells (A), breast cancer tumor (B) and fibroblasts (C) of index patient UPN 6144 (c.1146delA) conducted with unmethylated-specific primers shows approximately the same spike pattern representing PCR products and oligonucleotide primers (blue spikes). By contrast, no PCR products could be generated with methylated-specific primers and DNA from $CD34^{+}$ leukemic cells (D), breast cancer tumor and fibroblasts (not shown), respectively.

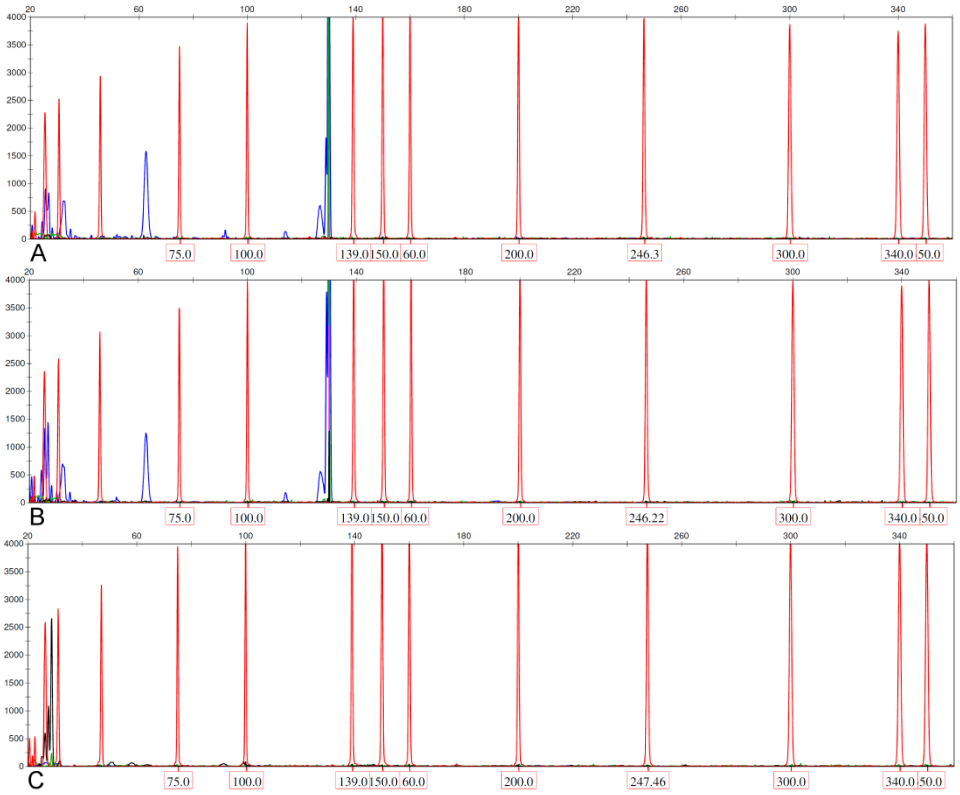

Figure 19. MSP of genomic DNA from breast cancer tumor (A) and fibroblasts (B) of index patient UPN 6371 shows PCR products and oligonucleotide primers (blue spikes) when conducted with unmethylated-specific primers, but not with methylated-specific primers (C, breast cancer).

3.6 P53 Immunohistochemistry

The principal behind p53 immunohistochemical staining is that mutant p53 is unable to transactivate MDM2 and thereby breaks a negative-feedback loop resulting in overexpression of p53 (Harris, Levine 2005). Mutant p53 is more stable than the normal protein and accumulates in the cell which can be visualized by staining (Vojtesek et al. 1992).

No bone marrow and leukemia samples were available in both index patients with germ-line *TP53* mutations (UPN 6371 and 6144) because they were used for extraction of DNA for mutational analyses. Likewise, breast cancer tissue of index patient UPN 6371 was unavailable for the same reason. Besides, p53 immunohistochemistry is not reliable in nonsense mutations like *TP53* c.849-852insGGCG (Alsner et al. 2008). Since *TP53* c.1146delA could also lead to a conformation change of the p53 protein, results should be

interpreted with care. Yet, to test pathologic transactivation of *TP53* c.1146delA, tumor samples of index patient UPN 6144, i.e. renal cell carcinoma and serous cystadenofibroma, were stained for increased accumulation of p53. P53 immunohistochemistry showed no pathologic staining in the renal cell carcinoma and little, insignificant staining of some Müllerian epithelial cells in the serous cystadenofibroma. Hence, p53 accumulation as an expression of complete loss of transactivation activity appears not to be important in the development of these tumors.

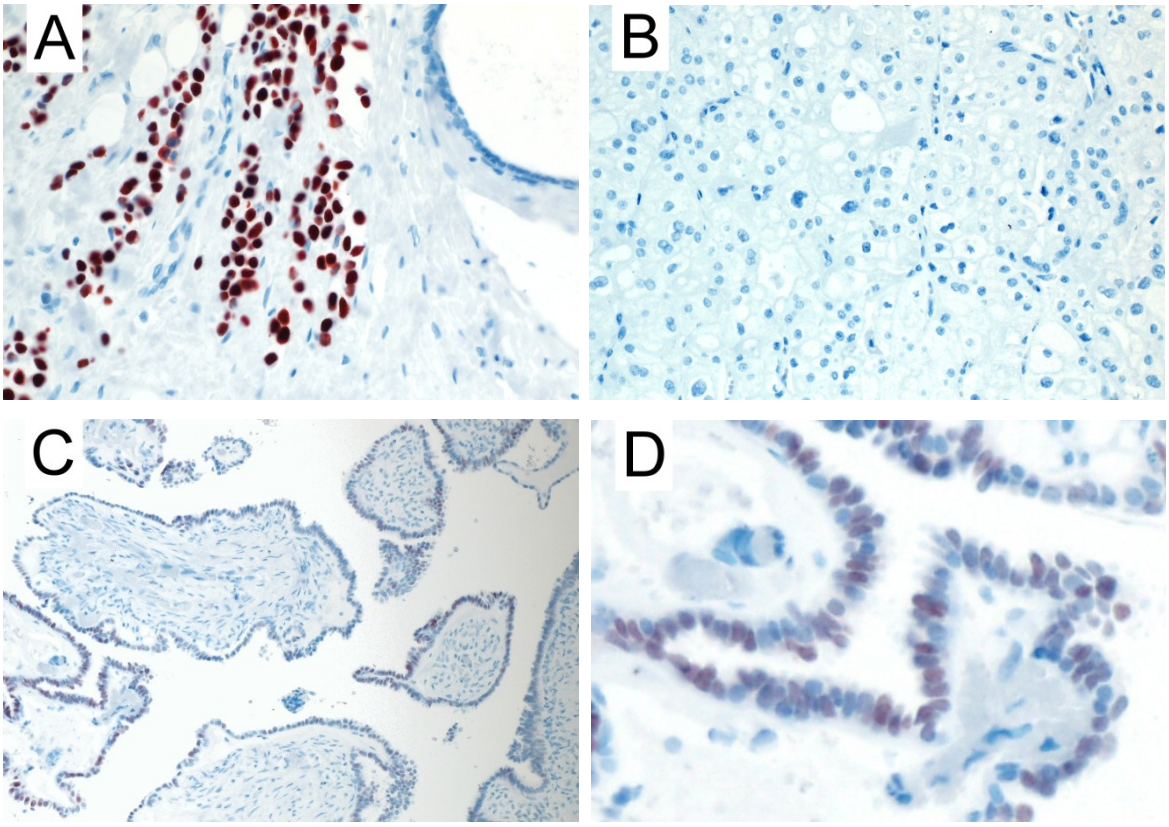

Figure 20. Immunohistochemical staining of p53 in tumors of index patient UPN 6144. The positive control (A) shows sharp brown staining of lobular carcinoma cells whereas healthy surrounding connective tissue and epithelial cells lining a cyst stay unstained. By contrast, patient´s renal cell carcinoma (B) shows no p53 staining and serous cystadenofibroma (C) indicates a vague staining in some Müllerian epithelial cells (D).

4 DISCUSSION

The present study focused on t-MN patients who received cytotoxic treatment for a malignant primary disease. From the initial sixty-nine patients with t-MNs, eight patients affected with autoimmune diseases were excluded on the assumption that they had a different genetic background compared to patients with neoplastic malignancies. The assumption was based on the hypothesis that in t-MN patients with neoplastic malignancies hereditary cancer syndromes were prevalent and contributed to the development of t-MNs through underlying germ-line mutations. One specific aim of the study was to indentify these patients.

The compilations of nuclear pedigrees led to the suspicion of genetic susceptibility to cancer in twelve index patients and eventually to the identification of five clinically relevant germ-line mutations in genes predisposing to cancer. Two of these mutations, *TP53* c.849-852insGGCG and c.1146delA, have not been previously described as germ-line mutations. To the best of the author´s knowledge, this study demonstrated for the first time the inactivation of the tumor suppressor *BRCA1* by LOH in therapy-related leukemic cells. Furthermore, it confirms the more recent finding of Link *et al*. who identified a LGR in the *TP53* gene by whole-genome sequencing in a patient with t-AML and showed that the wild-type allele was lost as a result of UPD in the leukemic clone (Link et al. 2011). Hence, this study gives additional preliminary evidence that germ-line mutations not only in *TP53* but also in *BRCA1* contribute to therapy-related leukemogenesis.

With the exception of *BARD1,* this study concentrated on mutations in genes leading to a rather salient phenotype, a heterogeneous tumor spectrum or a prominent family history of cancer because it was aimed to show that identification of patients with germ-line mutations was achievable by clinical criteria. The finding in this t-MN cohort that breast cancer was the most frequent malignancy with an association to genetic susceptibility became obvious during the pedigree compilation process and led to focus on genes predisposing to HBOCS. There are several genes which were not considered for different reasons. Genes highly associated with breast cancer like *NF1*, *STK11* (Peutz–Jeghers syndrome) and *NBC* (Nijmegen breakage syndrome) were not checked because cardinal features of respective syndromes like café-au-lait spots in NF1, harmatomas in Peutz–Jeghers syndrome and growth retardation in Nijmegen breakage syndrome were

not present in patients of this study. Rare, moderate-penetrance breast cancer susceptibility genes like *ATM* (Ataxia telangiectasia [A-T], Louis–Bar syndrome), *BRIP1* (*FANCJ*), *CHEK2*, *PALB2* (*FANCN*) and *RAD50* were not analyzed because with the exception of *ATM* they are not associated with a distinct phenotype allowing to identify mutation carriers (Ripperger et al. 2009). Again, no patients were found to show neurologic symptoms characteristic of A-T like early-onset progressive cerebellar ataxia.

The genes in which germ-line mutations were found are directly or indirectly involved in DNA repair and apoptosis, two important antioncogenic mechanisms deranged in t-MNs. For instance, chromosomal instability is frequent in t-MNs and could be an expression of impaired DNA repair which, in its functioning form, is particularly important for healthy tissues during cytotoxic treatments where direct DNA damage occurs. MDS is characterized by excessive apoptosis whereas AML shows lack of apoptosis and instead a differentiation block (Corey et al. 2007). Nevertheless, functional consequences of these mutations in myeloid cells and mechanisms perpetuating leukemogenesis are unknown. Besides, a causal relationship between these mutations and leukemogenesis is still unproven although the finding of LOH is in accord with the common paradigm of tumor suppressor silencing. Thus, in vitro studies must determine the pathogenicity of these germ-line mutations where reactions to cytotoxic agents are particularly interesting. The role of *BRCA1* in myeloid malignancies is poorly understood but biallelic germ-line mutations in *BRCA2*, also known as *FANCD1*, lead to the development of Fanconi anemia, a rare autosomal recessive disease with the propensity to develop AML (Howlett et al. 2002). On the contrary, somatic *TP53* mutations were shown to be common in t-MNs and to be associated with a poor prognosis (Christiansen, Andersen & Pedersen-Bjergaard 2001). Furthermore, *TP53* alterations have been related with disease progression in del(5q) MDS and with transformation of myeloproliferative neoplasms to AML (Jadersten et al. 2011, Harutyunyan et al. 2011).

The fact that *BRCA1* c.5251C>T and *TP53* c.1146delA did not show LOH in $CD34^+$ leukemic cells requires explanation. Although biallelic *BRCA* alterations are thought to be necessary in order to promote tumorigenesis, the recent finding of Bellacosa *et al*. indicates that malignant transformation begins as early as one *BRCA* allele is altered (Bellacosa et al. 2010). By comparing transcriptomes of phenotypically benign primary

breast and ovarian epithelial cultures of patients with monoallelic *BRCA1* and *BRCA2* mutations with cultures of healthy individuals they could show that mutated cells showed different expression profiles toward genes implicated in cancer. Interestingly, monoallelic *BRCA1* mutated breast cells showed an expression profile similar to those of mammary stem and progenitor cells. Although these results apply to epithelial cells and are not directly applicable to myeloid cells, they argue for an oncogenic effect of heterozygous *BRCA1* and together with the results of this thesis confirm the need for more studies in myeloid malignancies. Indeed, there is evidence that reduced *BRCA1* mRNA expression has a possible role in the development of t-MNs since Voso *et al*. could previously show that *BRCA1* under-expression due to promoter hypermethylation is not only frequent in AML but also highly associated with the therapy-related subtype (76 vs 31%, P=0.0002) (Scardocci et al. 2006). As we performed only a qualitative but no quantitative assessment of *BRCA1* cDNA, it cannot be ruled out that - although mRNA was admittedly heterozygous for c.5251C>T in $CD34^+$ leukemic blasts – the expression of the wild-type copy was lower compared to the mutated allele or that *BRCA1* expression was reduced at all. Therefore, reduced *BRCA1* expression could be another explanation for heterozygous c.5251C>T in $CD34^+$ leukemic blasts.

The lack of homozygous *TP53* c.1146delA in breast cancer and the negative p53 staining in the renal cell carcinoma as well as ovarian tumor are problematic as they raise the question whether *TP53* c.1146delA is a deleterious mutation in the first place. In cancers, somatic mutations of *TP53* lead frequently to a loss of function characterized by a complete loss of transactivation activity (TA) (Petitjean et al. 2007). Mutations that perturb p53 TA are found at CpG sites within the DNA binding domain encompassing exons 5 to 8 which are also hot spots for germ-line mutations (Varley 2003, Whibley, Pharoah & Hollstein 2009). In index patient UPN 6144, *TP53* promoters were not silenced by methylation in breast cancer and leukemic cells, and p53 immunohistochemical staining showed no pathologic accumulation in the renal cell carcinoma as well as serous cystadenofibroma. Assumed that p53 staining was not interfered by a protein conformation change and both *TP53* alleles were translated (which unfortunately could not be tested) then TA of *TP53* c.1146delA should be normal as was indicated by the lack of pathologic accumulation of p53. Maintained TA could also explain why the index

patient with *TP53* c.1146delA - besides pure coincidence - showed multiple neoplasms but late onset of malignant diseases. By comparing the mean age at onset of cancers with the transactivation property of confirmed germ-line *TP53* mutations in the IARC TP53 database Hainaut *et al*. have recently shown that the mean age at onset of breast as well as colon cancers was significantly lower in carriers with a mutation that led to a non-functional TA compared to individuals with a partially functional mutation (Petitjean et al. 2007). They concluded that the penetrance of a mutation may be related to its degree of loss of TA. These results are supported by a previous family study of LFS patients showing younger cancer manifestation in those with missense mutations in the DNA binding domain than in those with other protein inactivating mutations (Birch et al. 1998). Since germ-line c.1146delA *TP53* mutation results in a prolonged p53 protein but leaves the transactivation side intact, late onset of malignant diseases could be explained by a functioning TA. If TA was intact, two additional mechanisms of disease contribution were hypothetically possible for heterozygous *TP53* c.1146delA in cancer: Dominant-negative effect postulates that during hetero-oligomerization mutant p53 interferes with wild-type p53 and suppresses its function to transactivate target genes. In contrast, dominant-gain-of-function refers to a wild-type p53 independent effect of a mutant p53 protein (Olive et al. 2004, Olivier et al. 2009). Nonetheless, the molecular effects of expressed p53 c.1146delA remain enigmatic without functional cell studies. However, *TP53* c.1146delA has interesting possible implications on the response to genotoxic stress linking this mutation with the development of t-MNs. If translated, *TP53* c.1146delA would lead to a lysine to alanine substitution at residue 382 which is an important acetylation site for the stabilization of p53 by recruitment of histone acetyltransferases through ATM after irradiation (Saito et al. 2002). Moreover, the prolongation of p53 would also lead to a loss of the phosphorylation site at serine 392. This phosphorylation site is highly conserved and is needed for the induction as well as stabilization of p53 after UV radiation and many other different stimuli (Cox, Meek 2010). Anyhow, the occurrence of multiple tumors in the index patient, the detection in a gastric cancer cell line as well as in EBV immortalized lymphoblasts and the prolongation with loss of acetylation and phosporylation sites speak strongly in favor that *TP53* c.1146delA is a deleterious mutation.

The implications of the BARD1 Cys557Ser alteration on hematologic malignancies are unknown but studies in high risk patients of HBOCS as well as in vitro studies with breast cancer cell lines give considerable evidence for a pathogenic role. Intriguingly, BARD1 mediates apoptosis through p53 and repression of BARD1 decreases sensitivity to the apoptosis-inducing drug doxorubicin independently of BRCA1 (Irminger-Finger et al. 2001). Moreover, concomitant depletion of BRCA1 and BARD1 disturbs G1/S checkpoint arrest through absent p53 phosphorylation in response to irradiation (Fabbro et al. 2004). As far as Cys557Ser is concerned, a previous study of 126 Finish index patients tested negative for *BRCA1/2* lesions reported for the first time that the frequency of the Cys557Ser alteration was found to be elevated in individuals with a familial history of cancer when compared to healthy controls (5.6 v 1.4%, p = 0.005) (Karppinen et al. 2004). Further studies confirmed this increased frequency and showed that BARD1 Cys557Ser is an independent risk factor for breast cancer that is confined to high risk female patients and probably does not modify *BRCA1/2*-associated cancer risk (Stacey et al. 2006, Karppinen et al. 2006, Spurdle et al. 2011). In transient expression and apoptosis assays, breast cancer cell lines transfected with the Cys557Ser variant showed reduced capability of growth suppression and apoptosis (Sauer, Andrulis 2005). Unfortunately, this variant could not be investigated in leukemic cells due to lack of appropriate samples but given the role of BARD1 in apoptosis induction after genotoxic stress studies investigating its role in leukemia and especially in t-MNs would be quiet worthwhile.

From a clinical point of view the present study highlights the utility of pedigree analysis in patients with t-MNs to identify clinically relevant germ-line mutations contributing to cancer predisposition. No *TP53* germ-line mutations were found in patients who fulfilled clinical criteria for LFS/LFL but this does not disapprove the applicability of these criteria (see Table 7). Assuming that *TP53* germ-line mutations contribute to therapy-related leukemogenesis, it is still possible that mutation carriers could be identified by these criteria in different cohorts. It should be kept in mind that approximately five percent of familial cases can be expected being false negative in the case of not meeting current clinical classification schemes for LFS/LFL (Gonzalez et al. 2009). The number of missed cases is certainly higher in patients with *de novo* mutations lacking familial clustering of cancer. The present facts suggest that both TP53 mutation

carriers in this study most likely missed LFS/LFL criteria because their mutations developed *de novo* as well as were not passed on to their children. This assumption is supported by both siblings and the children of the index patient with *TP53* c.849-852insGGCG carrying no mutation and her parents showing no LFS/LFL typical tumors. Moreover, pedigree analysis of the index patient with *TP53* c.1146delA indicated no cancers in her relatives. In a large study of 525 patients submitted for *TP53* testing two out of seventy-five (2.7%) *TP53* mutation carriers with available family history data had leukemia (2.7%) and both had multiple primary cancers (breast cancer and sarcoma) (Gonzalez et al. 2009). It is unknown whether these leukemia cases were therapy-related but the notion of multiple primary cancers is important because it shows a potential identification criterion since at least one of the *TP53* mutations carriers in the present study had multiple malignant diseases in addition to t-MN.

Unlike LFS/LFL criteria German national Step 3 guideline (see Table 8) for early detection of breast cancer reconfirmed its utility in patients with t-MNs in this study since two *BRCA1* germ-line mutations were identified in six individuals tested (2/6; 33%).

Whether the results of this study apply to t-MN patients as a whole (5/51; 9.8%) or only to a certain subgroup is still unclear but it is striking that all t-MN patients with germ-line mutations had either solid tumors (5/26; 19.2%) or tumors belonging to the hereditary breast and ovarian cancer syndrome (5/14; 35.7%), respectively. Given the estimate that roughly 5% of breast cancers arise in the context of hereditary cancer predisposition syndromes, deleterious germ-line mutations are found with increased prevalence in this cohort of t-MN patients with breast cancer as primary diagnosis (4/13; 30.8%) (Ripperger et al. 2009). The increased risk of t-MNs after adjuvant treatment of breast cancer with alkylating agents or particularly with radiation is well established but the role of germ-line mutations in this setting is not defined (Smith et al. 2003a). With regard to genetic susceptibility to t-MNs it could be hypothesized that patients with hematologic malignancies and patients with solid tumors differ in their genetic background and as a result in the mechanisms leading to t-MN development.

The results of this study have important clinical relevance for families of t-MN patients with germ-line mutations in terms of therapeutic, preventive, and surveillance

considerations for relatives found to carry a mutation. *BRCA* mutation carriers diagnosed with breast cancer are at increased risk of developing contralateral breast cancer. Studies revealed that the risk is about 30% at 10 years and 47% at 25 years (Metcalfe et al. 2004, Graeser et al. 2009). Although studies have failed to show a significant overall survival difference between *BRCA*-associated and sporadic breast cancers for conventional therapy regimes (Bordeleau, Panchal & Goodwin 2010), there is considerable evidence that *BRCA* mutation carriers benefit from risk-reducing surgery (RRS), i.e. bilateral mastectomy (RRM) and salpingo-oophorectomy (RRSO). In a recent prospective study of *BRCA* mutation carriers Rebbeck *et al*. could show that RRSO in *BRCA* mutation carriers with or without prior breast cancer is associated with lower all-cause mortality (10% vs 3%), breast cancer–specific mortality (6% vs 2%), and ovarian cancer–specific mortality (3% vs 0.4%) compared with women who did not receive RRSO (Domchek et al. 2010). In a previous study by Rebbeck *et al*. RRM prior to breast cancer was shown to reduce the risk of breast cancer by approximately 90% in *BRCA* mutation carriers (Rebbeck et al. 2004). Currently, there are no prospective studies indicating that *TP53* mutation carriers benefit from close surveillance strategies in terms of overall survival (Lammens et al. 2010). Lack of appropriate data arises from heterogeneity and multiplicity of cancers associated with *TP53* germ-line mutations, insufficient number of individuals as well as unsettled optimal screening methods. However, F18-fluorodeoxyglucose–positron emission tomography/computed tomography (18-FDG-PET/CT) has recently shown promising results as a screening option for potentially treatable cancers in *TP53* mutation carriers (Masciari et al. 2008). Since breast cancer is the most frequent malignancy observed in *TP53* mutation carriers and affected premenopausal women are at highest risk of developing breast cancer, female *TP53* mutation carriers - like *BRCA* mutation carriers - require careful surveillance strategies (Gonzalez et al. 2009, Ruijs et al. 2010). At the moment magnetic resonance imaging (MRI) is regarded as the screening method of choice for the early detection of breast cancer in premenopausal patients with *TP53* germ-line mutations as well as *BRCA* mutation carriers and should be offered to all patients at high risk (Kriege et al. 2004, Leach et al. 2005). It has been argued that *TP53* mutation carriers who developed breast cancer should be informed about the option of prophylactic bilateral mastectomy and subsequent reconstruction because the risk of not only radio-induced malignancies but also contralateral breast cancers seems to be

increased (Heymann et al. 2010). Nevertheless, large prospective studies of patients with *BRCA1* as well as *TP53* germ-line mutations are needed to establish appropriate risk assessments of cytotoxic treatments and best therapy options including preference of RRS over cytotoxic therapy.

The possible contribution of germ-line mutations in therapy-related leukemogenesis demonstrated in this study calls for alternative therapy strategies in these patients. Inhibition of poly-adenosine diphosphate [ADP]–ribose polymerase (PARP) confers a promising new therapeutic approach by targeting specifically BRCA deficient breast cancer cells (Bryant et al. 2005). Cells rely on PARP to repair DNA single-strand breaks by base excision repair and inhibition of PARP forces DNA repair by homologous recombination through the BRCA pathway. Since breast cancer cells of *BRCA* mutation carriers have a dysfunctional BRCA pathway, genetic instability, cell cycle arrest and subsequent apoptosis occurs in the context of PARP inhibition (Farmer et al. 2005). This effect is selective for BRCA deficient breast cancer cells because healthy tissues still retain the wild-type allele. Two international, multicentre, phase 2 studies have recently shown the efficacy of PARP inhibitor Olaparib in advanced breast cancer and in recurrent ovarian cancer of BRCA mutation carriers (Tutt et al. 2010, Audeh et al. 2010). In these studies with patients who were pretreated and had poor prognoses tumor response rates were 41% for patients with breast cancer and 33% for patients with ovarian cancer, respectively, while no unique toxic effects were noted due to selective killing of cancer cells. For both patient groups overall response rate was 52% and median progression-free survival was approximately 6 months. Unfortunately, such promising therapy prospects are not on the horizon for *TP53* mutation carriers at present because no other known protein exists that can replace dysfunctional or missing p53 in cancer cells making prevention of primary and secondary malignancies in LFS/LFL patients essential (Ventura et al. 2007). Given the efficacy of PARP inhibitors in BRCA deficient cells there is a need for studies evaluating the prevalence of somatic *BRCA* mutations in t-MNs because it has recently been shown that PARP inhibitors are able to induce apoptosis in myeloid cells in vitro (Gaymes et al. 2009). Thus, knowing the *BRCA* mutation status in t-MNs could also facilitate personalized medicine of these myeloid malignancies.

Given the results of the pedigree analyses and the goal not to oversee patients and their respective families at risk for cancer, it is valid to propose that physicians should pay close attention to t-MN patients fulfilling clinical criteria of cancer syndromes, having young age of disease manifestation or showing multiple cancers, respectively. It is noteworthy that none of the patients with germ-line mutations have been suspected of having cancer syndromes by their caring physicians.

In sum, the present study shows that pedigree analysis is a good tool for the identification of hereditary cancer predisposition syndromes in patients with t-MNs. Furthermore, it gives preliminary evidence that germ-line mutations in genes causing these syndromes contribute to the development of t-MNs. Therefore, it is indicated to evaluate how strong the contribution is and whether different therapy and prevention strategies are needed for mutation carriers. The results of this study argue that the prevalence of hereditary cancer predisposition syndromes is increased in t-MN patients. Hence, the author proposes that pedigree analysis should be part of a general work up of new diagnosed t-MN patients because its results have important clinical implications for their families. Nevertheless, functional in vitro and even animal studies are needed to assess the pathogenic effects in t-MNs resulting from mutations in *BRCA1*, *BARD1* and *TP53* and to establish their role in therapy-related leukemogenesis. Time will tell if promising approaches like whole genome sequencing will simplify detection of cancer susceptibility genes in the context of t-MN and enable superior targeted therapies.

5 References

Affymetrix 2009, *Data Sheet: Genome-Wide Human SNP Array 6.0*, P/N 702509 Rev. 2 edn.

Alsner, J., Jensen, V., Kyndi, M., Offersen, B.V., Vu, P., Borresen-Dale, A.L. & Overgaard, J. 2008, "A comparison between p53 accumulation determined by immunohistochemistry and TP53 mutations as prognostic variables in tumours from breast cancer patients", *Acta Oncologica (Stockholm, Sweden),* vol. 47, no. 4, pp. 600-607.

Anensen, N., Skavland, J., Stapnes, C., Ryningen, A., Borresen-Dale, A.L., Gjertsen, B.T. & Bruserud, O. 2006, "Acute myelogenous leukemia in a patient with Li-Fraumeni syndrome treated with valproic acid, theophyllamine and all-trans retinoic acid: a case report", *Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, U.K,* vol. 20, no. 4, pp. 734-736.

Audeh, M.W., Carmichael, J., Penson, R.T., Friedlander, M., Powell, B., Bell-McGuinn, K.M., Scott, C., Weitzel, J.N., Oaknin, A., Loman, N., Lu, K., Schmutzler, R.K., Matulonis, U., Wickens, M. & Tutt, A. 2010, "Oral poly(ADP-ribose) polymerase inhibitor olaparib in patients with BRCA1 or BRCA2 mutations and recurrent ovarian cancer: a proof-of-concept trial", *Lancet,* vol. 376, no. 9737, pp. 245-251.

Bellacosa, A., Godwin, A.K., Peri, S., Devarajan, K., Caretti, E., Vanderveer, L., Bove, B., Slater, C., Zhou, Y., Daly, M., Howard, S., Campbell, K.S., Nicolas, E., Yeung, A.T., Clapper, M.L., Crowell, J.A., Lynch, H.T., Ross, E., Kopelovich, L. & Knudson, A.G. 2010, "Altered gene expression in morphologically normal epithelial cells from heterozygous carriers of BRCA1 or BRCA2 mutations", *Cancer prevention research (Philadelphia, Pa.),* vol. 3, no. 1, pp. 48-61.

Bennett, J.M., Catovsky, D., Daniel, M.T., Flandrin, G., Galton, D.A.G., Gralnick, H.R. & Sultan, C. 1985, "Proposed Revised Criteria for the Classification of Acute Myeloid-Leukemia - a Report of the French-American-British Cooperative Group", *Annals of Internal Medicine,* vol. 103, no. 4, pp. 620-625.

Ben-Yehuda, D., Krichevsky, S., Caspi, O., Rund, D., Polliack, A., Abeliovich, D., Zelig, O., Yahalom, V., Paltiel, O., Or, R., Peretz, T., Ben-Neriah, S., Yehuda, O. & Rachmilewitz, E.A. 1996, "Microsatellite instability and p53 mutations in therapy-related leukemia suggest mutator phenotype", *Blood,* vol. 88, no. 11, pp. 4296-4303.

Birch, J.M., Blair, V., Kelsey, A.M., Evans, D.G., Harris, M., Tricker, K.J. & Varley, J.M. 1998, "Cancer phenotype correlates with constitutional TP53 genotype in families with the Li-Fraumeni syndrome", *Oncogene,* vol. 17, no. 9, pp. 1061-1068.

Birch, J.M., Hartley, A.L., Tricker, K.J., Prosser, J., Condie, A., Kelsey, A.M., Harris, M., Jones, P.H., Binchy, A. & Crowther, D. 1994, "Prevalence and diversity of

constitutional mutations in the p53 gene among 21 Li-Fraumeni families", *Cancer research,* vol. 54, no. 5, pp. 1298-1304.

Bordeleau, L., Panchal, S. & Goodwin, P. 2010, "Prognosis of BRCA-associated breast cancer: a summary of evidence", *Breast cancer research and treatment,* vol. 119, no. 1, pp. 13-24.

Brody, J.R. & Kern, S.E. 2004, "History and principles of conductive media for standard DNA electrophoresis", *Analytical Biochemistry,* vol. 333, no. 1, pp. 1-13.

Bryant, H.E., Schultz, N., Thomas, H.D., Parker, K.M., Flower, D., Lopez, E., Kyle, S., Meuth, M., Curtin, N.J. & Helleday, T. 2005, "Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase", *Nature,* vol. 434, no. 7035, pp. 913-917.

Bullinger, L., Ehrich, M., Dohner, K., Schlenk, R.F., Dohner, H., Nelson, M.R. & van den Boom, D. 2010, "Quantitative DNA methylation predicts survival in adult acute myeloid leukemia", *Blood,* vol. 115, no. 3, pp. 636-642.

Burnett, A., Wetzler, M. & Lowenberg, B. 2011, "Therapeutic advances in acute myeloid leukemia", *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* vol. 29, no. 5, pp. 487-494.

Centers for Disease Control and Prevention (CDC) 2011, "Cancer survivors --- United States, 2007 ", *MMWR.Morbidity and mortality weekly report,* vol. 60, no. 9, pp. 269-272.

Chao, R.C., Pyzel, U., Fridlyand, J., Kuo, Y.M., Teel, L., Haaga, J., Borowsky, A., Horvai, A., Kogan, S.C., Bonifas, J., Huey, B., Jacks, T.E., Albertson, D.G. & Shannon, K.M. 2005, "Therapy-induced malignant neoplasms in Nf1 mutant mice", *Cancer cell,* vol. 8, no. 4, pp. 337-348.

Chen, J., Odenike, O. & Rowley, J.D. 2010, "Leukaemogenesis: more than mutant genes", *Nature reviews.Cancer,* vol. 10, no. 1, pp. 23-36.

Cheson, B.D., Bennett, J.M., Kopecky, K.J., Buchner, T., Willman, C.L., Estey, E.H., Schiffer, C.A., Doehner, H., Tallman, M.S., Lister, T.A., Lo-Coco, F., Willemze, R., Biondi, A., Hiddemann, W., Larson, R.A., Lowenberg, B., Sanz, M.A., Head, D.R., Ohno, R., Bloomfield, C.D. & International Working Group for Diagnosis, Standardization of Response Criteria, Treatment Outcomes, and Reporting Standards for Therapeutic Trials in Acute Myeloid Leukemia 2003, "Revised recommendations of the International Working Group for Diagnosis, Standardization of Response Criteria, Treatment Outcomes, and Reporting Standards for Therapeutic Trials in Acute Myeloid Leukemia", *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* vol. 21, no. 24, pp. 4642-4649.

Chompret, A., Abel, A., Stoppa-Lyonnet, D., Brugieres, L., Pages, S., Feunteun, J. & Bonaiti-Pellie, C. 2001, "Sensitivity and predictive value of criteria for p53 germline mutation screening", *Journal of medical genetics,* vol. 38, no. 1, pp. 43-47.

Christiansen, D.H., Andersen, M.K. & Pedersen-Bjergaard, J. 2001, "Mutations with loss of heterozygosity of p53 are common in therapy-related myelodysplasia and acute myeloid leukemia after exposure to alkylating agents and significantly associated with deletion or loss of 5q, a complex karyotype, and a poor prognosis", *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* vol. 19, no. 5, pp. 1405-1413.

Corey, S.J., Minden, M.D., Barber, D.L., Kantarjian, H., Wang, J.C. & Schimmer, A.D. 2007, "Myelodysplastic syndromes: the complexity of stem-cell diseases", *Nature reviews.Cancer,* vol. 7, no. 2, pp. 118-129.

Cox, M.L. & Meek, D.W. 2010, "Phosphorylation of serine 392 in p53 is a common and integral event during p53 induction by diverse stimuli ", *Cellular signalling,* vol. 22, no. 3, pp. 564-571.

Craig, F.E. & Foon, K.A. 2008, "Flow cytometric immunophenotyping for hematologic neoplasms", *Blood,* vol. 111, no. 8, pp. 3941-3967.

Delhommeau, F., Dupont, S., Della Valle, V., James, C., Trannoy, S., Masse, A., Kosmider, O., Le Couedic, J.P., Robert, F., Alberdi, A., Lecluse, Y., Plo, I., Dreyfus, F.J., Marzac, C., Casadevall, N., Lacombe, C., Romana, S.P., Dessen, P., Soulier, J., Viguie, F., Fontenay, M., Vainchenker, W. & Bernard, O.A. 2009, "Mutation in TET2 in myeloid cancers", *The New England journal of medicine,* vol. 360, no. 22, pp. 2289-2301.

Dockhorn-Dworniczak, B., Wolff, J., Poremba, C., Schafer, K.L., Ritter, J., Gullotta, F., Jurgens, H. & Bocker, W. 1996, "A new germline TP53 gene mutation in a family with Li-Fraumeni syndrome", *European journal of cancer (Oxford, England : 1990),* vol. 32A, no. 8, pp. 1359-1365.

Doehner, H., Estey, E.H., Amadori, S., Appelbaum, F.R., Buechner, T., Burnett, A.K., Dombret, H., Fenaux, P., Grimwade, D., Larson, R.A., Lo-Coco, F., Naoe, T., Niederwieser, D., Ossenkoppele, G.J., Sanz, M.A., Sierra, J., Tallman, M.S., Loewenberg, B. & Bloomfield, C.D. 2010, "Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet", *Blood,* vol. 115, no. 3, pp. 453-474.

Domchek, S.M., Friebel, T.M., Singer, C.F., Evans, D.G., Lynch, H.T., Isaacs, C., Garber, J.E., Neuhausen, S.L., Matloff, E., Eeles, R., Pichert, G., Van t'veer, L., Tung, N., Weitzel, J.N., Couch, F.J., Rubinstein, W.S., Ganz, P.A., Daly, M.B., Olopade, O.I., Tomlinson, G., Schildkraut, J., Blum, J.L. & Rebbeck, T.R. 2010, "Association of risk-reducing surgery in BRCA1 or BRCA2 mutation carriers with cancer risk and mortality", *JAMA : the journal of the American Medical Association,* vol. 304, no. 9, pp. 967-975.

Ebert, B.L., Pretz, J., Bosco, J., Chang, C.Y., Tamayo, P., Galili, N., Raza, A., Root, D.E., Attar, E., Ellis, S.R. & Golub, T.R. 2008, "Identification of RPS14 as a 5q- syndrome gene by RNA interference screen", *Nature,* vol. 451, no. 7176, pp. 335-339.

Eeles, R.A. 1995, "Germline mutations in the TP53 gene", *Cancer surveys,* vol. 25, pp. 101-124.

Engert, S., Wappenschmidt, B., Betz, B., Kast, K., Kutsche, M., Hellebrand, H., Goecke, T.O., Kiechle, M., Niederacher, D., Schmutzler, R.K. & Meindl, A. 2008, "MLPA screening in the BRCA1 gene from 1,506 German hereditary breast cancer cases: novel deletions, frequent involvement of exon 17, and occurrence in single early-onset cases", *Human mutation,* vol. 29, no. 7, pp. 948-958.

Fabbro, M., Savage, K., Hobson, K., Deans, A.J., Powell, S.N., McArthur, G.A. & Khanna, K.K. 2004, "BRCA1-BARD1 complexes are required for p53Ser-15 phosphorylation and a G1/S arrest following ionizing radiation-induced DNA damage", *The Journal of biological chemistry,* vol. 279, no. 30, pp. 31251-31258.

Farmer, H., McCabe, N., Lord, C.J., Tutt, A.N., Johnson, D.A., Richardson, T.B., Santarosa, M., Dillon, K.J., Hickson, I., Knights, C., Martin, N.M., Jackson, S.P., Smith, G.C. & Ashworth, A. 2005, "Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy", *Nature,* vol. 434, no. 7035, pp. 917-921.

Felix, C.A., Hosler, M.R., Provisor, D., Salhany, K., Sexsmith, E.A., Slater, D.J., Cheung, N.K., Winick, N.J., Strauss, E.A., Heyn, R., Lange, B.J. & Malkin, D. 1996, "The p53 gene in pediatric therapy-related leukemia and myelodysplasia", *Blood,* vol. 87, no. 10, pp. 4376-4381.

Figueroa, M.E., Abdel-Wahab, O., Lu, C., Ward, P.S., Patel, J., Shih, A., Li, Y., Bhagwat, N., Vasanthakumar, A., Fernandez, H.F., Tallman, M.S., Sun, Z., Wolniak, K., Peeters, J.K., Liu, W., Choe, S.E., Fantin, V.R., Paietta, E., Lowenberg, B., Licht, J.D., Godley, L.A., Delwel, R., Valk, P.J., Thompson, C.B., Levine, R.L. & Melnick, A. 2010, "Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation", *Cancer cell,* vol. 18, no. 6, pp. 553-567.

Figueroa, M.E., Skrabanek, L., Li, Y., Jiemjit, A., Fandy, T.E., Paietta, E., Fernandez, H., Tallman, M.S., Greally, J.M., Carraway, H., Licht, J.D., Gore, S.D. & Melnick, A. 2009, "MDS and secondary AML display unique patterns and abundance of aberrant DNA methylation", *Blood,* vol. 114, no. 16, pp. 3448-3458.

Flotho, C., Steinemann, D., Mullighan, C.G., Neale, G., Mayer, K., Kratz, C.P., Schlegelberger, B., Downing, J.R. & Niemeyer, C.M. 2007, "Genome-wide single-nucleotide polymorphism analysis in juvenile myelomonocytic leukemia identifies uniparental disomy surrounding the NF1 locus in cases associated with neurofibromatosis but not in cases with mutant RAS or PTPN11", *Oncogene,* vol. 26, no. 39, pp. 5816-5821.

Gardner, L.B. 2010, "Nonsense-mediated RNA decay regulation by cellular stress: implications for tumorigenesis", *Molecular cancer research : MCR,* vol. 8, no. 3, pp. 295-308.

Gaymes, T.J., Shall, S., MacPherson, L.J., Twine, N.A., Lea, N.C., Farzaneh, F. & Mufti, G.J. 2009, "Inhibitors of poly ADP-ribose polymerase (PARP) induce apoptosis of myeloid leukemic cells: potential for therapy of myeloid leukemia and myelodysplastic syndromes", *Haematologica,* vol. 94, no. 5, pp. 638-646.

Ginsberg, J.P., de Alava, E., Ladanyi, M., Wexler, L.H., Kovar, H., Paulussen, M., Zoubek, A., Dockhorn-Dworniczak, B., Juergens, H., Wunder, J.S., Andrulis, I.L., Malik, R., Sorensen, P.H., Womer, R.B. & Barr, F.G. 1999, "EWS-FLI1 and EWS-ERG gene fusions are associated with similar clinical phenotypes in Ewing's sarcoma ", *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* vol. 17, no. 6, pp. 1809-1814.

Gonzalez, K.D., Noltner, K.A., Buzin, C.H., Gu, D., Wen-Fong, C.Y., Nguyen, V.Q., Han, J.H., Lowstuter, K., Longmate, J., Sommer, S.S. & Weitzel, J.N. 2009, "Beyond Li Fraumeni Syndrome: clinical characteristics of families with p53 germline mutations", *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* vol. 27, no. 8, pp. 1250-1256.

Graeser, M.K., Engel, C., Rhiem, K., Gadzicki, D., Bick, U., Kast, K., Froster, U.G., Schlehe, B., Bechtold, A., Arnold, N., Preisler-Adams, S., Nestle-Kraemling, C., Zaino, M., Loeffler, M., Kiechle, M., Meindl, A., Varga, D. & Schmutzler, R.K. 2009, "Contralateral breast cancer risk in BRCA1 and BRCA2 mutation carriers", *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* vol. 27, no. 35, pp. 5887-5892.

Greenberg, P., Cox, C., LeBeau, M., Fenaux, P., Morel, P., Sanz, G., Sanz, M., Vallespi, T., Hamblin, T., Oscier, D., Ohyashiki, K., Toyama, K., Aul, C., Mufti, G. & Bennett, J. 1997, "International scoring system for evaluating prognosis in myelodysplastic syndromes", *Blood,* vol. 89, no. 6, pp. 2079-2088.

Greenberg, P.L. 2010, "Current therapeutic approaches for patients with myelodysplastic syndromes", *British journal of haematology,* vol. 150, no. 2, pp. 131-143.

Grimwade, D. & Hills, R.K. 2009, "Independent prognostic factors for AML outcome", *Hematology / the Education Program of the American Society of Hematology.American Society of Hematology.Education Program,* , pp. 385-395.

Haase, D., Germing, U., Schanz, J., Pfeilstoecker, M., Noesslinger, T., Hildebrandt, B., Kundgen, A., Luebbert, M., Kunzmann, R., Giagounidis, A.A.N., Aul, C., Truemper, L., Krieger, O., Stauder, R., Mueller, T.H., Wimazal, F., Valent, P., Fonatsch, C. & Steidl, C. 2007, "New insights into the prognostic impact of the karyotype in MDS and correlation with subtypes: evidence from a core dataset of 2124 patients", *Blood,* vol. 110, no. 13, pp. 4385-4395.

Hanahan, D. & Weinberg, R.A. 2011, "Hallmarks of cancer: the next generation", *Cell,* vol. 144, no. 5, pp. 646-674.

Harris, S.L. & Levine, A.J. 2005, "The p53 pathway: positive and negative feedback loops", *Oncogene,* vol. 24, no. 17, pp. 2899-2908.

Harutyunyan, A., Klampfl, T., Cazzola, M. & Kralovics, R. 2011, "P53 Lesions in Leukemic Transformation", *The New England journal of medicine,* vol. 364, no. 5, pp. 488-490.

Herman, J.G., Graff, J.R., Myohanen, S., Nelkin, B.D. & Baylin, S.B. 1996, "Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands ", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 93, no. 18, pp. 9821-9826.

Heymann, S., Delaloge, S., Rahal, A., Caron, O., Frebourg, T., Barreau, L., Pachet, C., Mathieu, M.C., Marsiglia, H. & Bourgier, C. 2010, "Radio-induced malignancies after breast cancer postoperative radiotherapy in patients with Li-Fraumeni syndrome", *Radiation oncology (London, England),* vol. 5, pp. 104.

Hisada, M., Garber, J.E., Fung, C.Y., Fraumeni, J.F.,Jr & Li, F.P. 1998, "Multiple primary cancers in families with Li-Fraumeni syndrome", *Journal of the National Cancer Institute,* vol. 90, no. 8, pp. 606-611.

Howlett, N.G., Taniguchi, T., Olson, S., Cox, B., Waisfisz, Q., De Die-Smulders, C., Persky, N., Grompe, M., Joenje, H., Pals, G., Ikeda, H., Fox, E.A. & D'Andrea, A.D. 2002, "Biallelic inactivation of BRCA2 in Fanconi anemia", *Science (New York, N.Y.),* vol. 297, no. 5581, pp. 606-609.

International HapMap Consortium. 2007, "A second generation human haplotype map of over 3.1 million SNPs", *Nature,* vol. 449, no. 7164, pp. 851-861.

Irminger-Finger, I., Leung, W.C., Li, J., Dubois-Dauphin, M., Harb, J., Feki, A., Jefford, C.E., Soriano, J.V., Jaconi, M., Montesano, R. & Krause, K.H. 2001, "Identification of BARD1 as mediator between proapoptotic stress and p53-dependent apoptosis", *Molecular cell,* vol. 8, no. 6, pp. 1255-1266.

Jacks, T., Shih, T.S., Schmitt, E.M., Bronson, R.T., Bernards, A. & Weinberg, R.A. 1994, "Tumor Predisposition in Mice Heterozygous for a Targeted Mutation in Nf1", *Nature genetics,* vol. 7, no. 3, pp. 353-361.

Jadersten, M., Saft, L., Smith, A., Kulasekararaj, A., Pomplun, S., Gohring, G., Hedlund, A., Hast, R., Schlegelberger, B., Porwit, A., Hellstrom-Lindberg, E. & Mufti, G.J. 2011, "TP53 Mutations in Low-Risk Myelodysplastic Syndromes With del(5q) Predict Disease Progression", *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* vol. 29, no. 15, pp. 1971-1979.

Janeway, C.A., Travers, P., Walport, M. & Shlomchik, M.J. 2001, "A-22 Flow cytometry and FACS analysis" in *Immunobiology*, 5th edn, Garland Science, New York.

Jasek, M., Gondek, L.P., Bejanyan, N., Tiu, R., Huh, J., Theil, K.S., O'Keefe, C., McDevitt, M.A. & Maciejewski, J.P. 2010, "TP53 mutations in myeloid malignancies are either

homozygous or hemizygous due to copy number-neutral loss of heterozygosity or deletion of 17p", *Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, U.K,* vol. 24, no. 1, pp. 216-219.

Juliusson, G., Antunovic, P., Derolf, A., Lehmann, S., Mollgard, L., Stockelberg, D., Tidefelt, U., Wahlin, A. & Hoglund, M. 2009, "Age and acute myeloid leukemia: real world data on decision to treat and outcomes from the Swedish Acute Leukemia Registry", *Blood,* vol. 113, no. 18, pp. 4179-4187.

Karppinen, S.M., Barkardottir, R.B., Backenhorn, K., Sydenham, T., Syrjakoski, K., Schleutker, J., Ikonen, T., Pylkas, K., Rapakko, K., Erkko, H., Johannesdottir, G., Gerdes, A.M., Thomassen, M., Agnarsson, B.A., Grip, M., Kallioniemi, A., Kere, J., Aaltonen, L.A., Arason, A., Moller, P., Kruse, T.A., Borg, A. & Winqvist, R. 2006, "Nordic collaborative study of the BARD1 Cys557Ser allele in 3956 patients with cancer: enrichment in familial BRCA1/BRCA2 mutation-negative breast cancer but not in other malignancies", *Journal of medical genetics,* vol. 43, no. 11, pp. 856-862.

Karppinen, S.M., Heikkinen, K., Rapakko, K. & Winqvist, R. 2004, "Mutation screening of the BARD1 gene: evidence for involvement of the Cys557Ser allele in hereditary susceptibility to breast cancer ", *Journal of medical genetics,* vol. 41, no. 9, pp. e114.

Kayser, S., Dohner, K., Krauter, J., Kohne, C.H., Horst, H.A., Held, G., von Lilienfeld-Toal, M., Wilhelm, S., Kundgen, A., Gotze, K., Rummel, M., Nachbaur, D., Schlegelberger, B., Gohring, G., Spath, D., Morlok, C., Zucknick, M., Ganser, A., Dohner, H., Schlenk, R.F. & German-Austrian AMLSG 2011, "The impact of therapy-related acute myeloid leukemia (AML) on outcome in 2853 adult patients with newly diagnosed AML", *Blood,* vol. 117, no. 7, pp. 2137-2145.

Kern, W., Haferlach, T., Schnittger, S., Hiddemann, W. & Schoch, C. 2004, "Prognosis in therapy-related acute myeloid leukemia and impact of karyotype", *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* vol. 22, no. 12, pp. 2510-2511.

King, M.C., Marks, J.H., Mandell, J.B. & New York Breast Cancer Study Group 2003, "Breast and ovarian cancer risks due to inherited mutations in BRCA1 and BRCA2", *Science (New York, N.Y.),* vol. 302, no. 5645, pp. 643-646.

Klenow, H. & Henningsen, I. 1970, "Selective elimination of the exonuclease activity of the deoxyribonucleic acid polymerase from Escherichia coli B by limited proteolysis ", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 65, no. 1, pp. 168-175.

Kreienberg, R., Kopp, I., Albert, U., Bartsch, H.H., Beckmann, M.W., Berg, D., Bick, U., du Bois, A., Budach, W., Dunst, J., Engel, J., Ernst, B., Geraedts, M., Henscher, U., Hölzel, D., Jackisch, C., König, K., Kreipe, H., Kühn, T., Lebeau, A., Leinung, S., Link, H., Lück, H.-., Madjar, H., Maiwald, A., Maiwald, G., Marschner, N., Marx, M., von Minckwitz, G., Naß-Griegoleit, I., Possinger, K., Reiter, A., Sauerbrei, W., Schlake, W., Schmutzler, R., Schreer, I., Schulte, H., Schulz, K.-., Souchon, R., Thomssen, C., Untch,

M., Wagner, U., Weis, J. & Zemmler, T. 2008, *Interdisziplinäre S3-Leitlinie für die Diagnostik, Therapie und Nachsorge des Mammakarzinoms,* 1. edn, W. Zuckschwerdt Verlag GmbH, Germering/München.

Kriege, M., Brekelmans, C.T.M., Boetes, C., Besnard, P.E., Zonderland, H.M., Obdeijn, I.M., Manoliu, R.A., Kok, T., Peterse, H., Tilanus-Linthorst, M.M.A., Muller, S.H., Meijer, S., Oosterwijk, J.C., Beex, L.V.A.M., Tollenaar, R.A.E.M., de Koning, H.J., Rutgers, E.J.T., Klijn, J.G.M. & Magnetic Resonance Imaging Screeni 2004, "Efficacy of MRI and mammography for breast-cancer screening in women with a familial or genetic predisposition", *New England Journal of Medicine,* vol. 351, no. 5, pp. 427-437.

Kumar, V., Abbas, A.K., Fausto, N. & Aster, J. 2010, *Robbins & Cotran Pathologic Basis of Disease,* 8th edn, Saunders/Elsevier, Philadelphia, PA.

Lammens, C.R., Bleiker, E.M., Aaronson, N.K., Wagner, A., Sijmons, R.H., Ausems, M.G., Vriends, A.H., Ruijs, M.W., van Os, T.A., Spruijt, L., Gomez Garcia, E.B., Cats, A., Nagtegaal, T. & Verhoef, S. 2010, "Regular surveillance for Li-Fraumeni Syndrome: advice, adherence and perceived benefits", *Familial cancer,* vol. 9, no. 4, pp. 647-654.

Langemeijer, S.M., Kuiper, R.P., Berends, M., Knops, R., Aslanyan, M.G., Massop, M., Stevens-Linders, E., van Hoogen, P., van Kessel, A.G., Raymakers, R.A., Kamping, E.J., Verhoef, G.E., Verburgh, E., Hagemeijer, A., Vandenberghe, P., de Witte, T., van der Reijden, B.A. & Jansen, J.H. 2009, "Acquired mutations in TET2 are common in myelodysplastic syndromes", *Nature genetics,* vol. 41, no. 7, pp. 838-842.

Lapidot, T., Sirard, C., Vormoor, J., Murdoch, B., Hoang, T., Caceres-Cortes, J., Minden, M., Paterson, B., Caligiuri, M.A. & Dick, J.E. 1994, "A cell initiating human acute myeloid leukaemia after transplantation into SCID mice ", *Nature,* vol. 367, no. 6464, pp. 645-648.

Leach, M.O., Boggis, C.R.M., Dixon, A.K., Easton, D.F., Eeles, R.A., Evans, D.G.R., Gilbert, F.F., Griebsch, I., Hoff, R.J.C., Kessar, P., Lakhani, S.R., Moss, S.M., Nerurkar, A., Padhani, A.R., Pointon, L.J., Thompson, D., Warren, R.M.L. & MARIBS Study Grp 2005, "Screening with magnetic resonance imaging and mammography of a UK population at high familial risk of breast cancer: a prospective multicentre cohort study (MARIBS)", *Lancet,* vol. 365, no. 9473, pp. 1769-1778.

Leone, G., Pagano, L., Ben-Yehuda, D. & Voso, M.T. 2007, "Therapy-related leukemia and myelodysplasia: susceptibility and incidence", *Haematologica,* vol. 92, no. 10, pp. 1389-1398.

Li, F.P., Fraumeni, J.F.,Jr, Mulvihill, J.J., Blattner, W.A., Dreyfus, M.G., Tucker, M.A. & Miller, R.W. 1988, "A cancer family syndrome in twenty-four kindreds", *Cancer research,* vol. 48, no. 18, pp. 5358-5362.

Link, D.C., Schuettpelz, L.G., Shen, D., Wang, J., Walter, M.J., Kulkarni, S., Payton, J.E., Ivanovich, J., Goodfellow, P.J., Le Beau, M., Koboldt, D.C., Dooling, D.J., Fulton, R.S.,

Bender, R.H., Fulton, L.L., Delehaunty, K.D., Fronick, C.C., Appelbaum, E.L., Schmidt, H., Abbott, R., O'Laughlin, M., Chen, K., McLellan, M.D., Varghese, N., Nagarajan, R., Heath, S., Graubert, T.A., Ding, L., Ley, T.J., Zambetti, G.P., Wilson, R.K. & Mardis, E.R. 2011, "Identification of a novel TP53 cancer susceptibility mutation through whole-genome sequencing of a patient with therapy-related AML", *JAMA : the journal of the American Medical Association,* vol. 305, no. 15, pp. 1568-1576.

Lowenberg, B., Downing, J. & Burnett, A. 1999, "Medical progress - Acute myeloid leukemia", *New England Journal of Medicine,* vol. 341, no. 14, pp. 1051-1062.

Lynch, H.T., Silva, E., Snyder, C. & Lynch, J.F. 2008, "Hereditary breast cancer: part I. Diagnosing hereditary breast cancer syndromes ", *The breast journal,* vol. 14, no. 1, pp. 3-13.

Mahgoub, N., Taylor, B.R., Le Beau, M.M., Gratiot, M., Carlson, K.M., Atwater, S.K., Jacks, T. & Shannon, K.M. 1999, "Myeloid malignancies induced by alkylating agents in Nf1 mice", *Blood,* vol. 93, no. 11, pp. 3617-3623.

Malcovati, L., Della Porta, M., Pascutto, C., Invernizzi, R., Boni, M., Travaglino, E., Passamonti, F., Arcaini, L., Maffioli, M., Bernasconi, P., Lazzarino, M. & Cazzola, M. 2005, "Prognostic factors and life expectancy in myelodysplastic syndromes classified according to WHO criteria: A basis for clinical decision making", *Journal of Clinical Oncology,* vol. 23, no. 30, pp. 7594-7603.

Malkin, D., Li, F.P., Strong, L.C., Fraumeni, J.F.,Jr, Nelson, C.E., Kim, D.H., Kassel, J., Gryka, M.A., Bischoff, F.Z. & Tainsky, M.A. 1990, "Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms", *Science (New York, N.Y.),* vol. 250, no. 4985, pp. 1233-1238.

Maris, J.M., Wiersma, S.R., Mahgoub, N., Thompson, P., Geyer, R.J., Hurwitz, C.G., Lange, B.J. & Shannon, K.M. 1997, "Monosomy 7 myelodysplastic syndrome and other second malignant neoplasms in children with neurofibromatosis type 1", *Cancer,* vol. 79, no. 7, pp. 1438-1446.

Masciari, S., Van den Abbeele, A.D., Diller, L.R., Rastarhuyeva, I., Yap, J., Schneider, K., Digianni, L., Li, F.P., Fraumeni, J.F.,Jr, Syngal, S. & Garber, J.E. 2008, "F18-fluorodeoxyglucose-positron emission tomography/computed tomography screening in Li-Fraumeni syndrome", *JAMA : the journal of the American Medical Association,* vol. 299, no. 11, pp. 1315-1319.

Mauritzson, N., Albin, M., Rylander, L., Billstrom, R., Ahlgren, T., Mikoczy, Z., Bjork, J., Stromberg, U., Nilsson, P.G., Mitelman, F., Hagmar, L. & Johansson, B. 2002, "Pooled analysis of clinical and cytogenetic features in treatment-related and de novo adult acute myeloid leukemia and myelodysplastic syndromes based on a consecutive series of 761 patients analyzed 1976-1993 and on 5098 unselected cases reported in the literature 1974-2001", *Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, U.K,* vol. 16, no. 12, pp. 2366-2378.

Metcalfe, K., Lynch, H.T., Ghadirian, P., Tung, N., Olivotto, I., Warner, E., Olopade, O.I., Eisen, A., Weber, B., McLennan, J., Sun, P., Foulkes, W.D. & Narod, S.A. 2004, "Contralateral breast cancer in BRCA1 and BRCA2 mutation carriers", *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* vol. 22, no. 12, pp. 2328-2335.

Mohamad, H.B. & Apffelstaedt, J.P. 2008, "Counseling for male BRCA mutation carriers: a review", *Breast (Edinburgh, Scotland),* vol. 17, no. 5, pp. 441-450.

Nichols, K.E., Heath, J.A., Friedman, D., Biegel, J.A., Ganguly, A., Mauch, P. & Diller, L. 2003, "TP53, BRCA1, and BRCA2 tumor suppressor genes are not commonly mutated in survivors of Hodgkin's disease with second primary neoplasms", *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* vol. 21, no. 24, pp. 4505-4509.

Nikoloski, G., Langemeijer, S.M., Kuiper, R.P., Knops, R., Massop, M., Tonnissen, E.R., van der Heijden, A., Scheele, T.N., Vandenberghe, P., de Witte, T., van der Reijden, B.A. & Jansen, J.H. 2010, "Somatic mutations of the histone methyltransferase gene EZH2 in myelodysplastic syndromes", *Nature genetics,* vol. 42, no. 8, pp. 665-667.

Noble, P.B. & Cutts, J.H. 1967, "Separation of blood leukocytes by Ficoll gradient ", *The Canadian veterinary journal.La revue veterinaire canadienne,* vol. 8, no. 5, pp. 110-111.

Olive, K.P., Tuveson, D.A., Ruhe, Z.C., Yin, B., Willis, N.A., Bronson, R.T., Crowley, D. & Jacks, T. 2004, "Mutant p53 gain of function in two mouse models of Li-Fraumeni syndrome", *Cell,* vol. 119, no. 6, pp. 847-860.

Olivier, M., Petitjean, A., Marcel, V., Petre, A., Mounawar, M., Plymoth, A., de Fromentel, C.C. & Hainaut, P. 2009, "Recent advances in p53 research: an interdisciplinary perspective", *Cancer gene therapy,* vol. 16, no. 1, pp. 1-12.

Owen, C., Barnett, M. & Fitzgibbon, J. 2008, "Familial myelodysplasia and acute myeloid leukaemia--a review", *British journal of haematology,* vol. 140, no. 2, pp. 123-132.

Pagana, L., Pulsoni, A., Tosti, M.E., Avvisati, G., Mele, L., Mele, M., Martino, B., Visani, G., Cerri, R., Di Bona, E., Invernizzi, R., Nosari, A., Clavio, M., Allione, B., Coser, P., Candoni, A., Levis, A., Camera, A., Melillo, L., Leone, G., Mandelli, F. & Gruppo Italiano Malattie Ematologiche Maligne dell'Adulto 2001, "Clinical and biological features of acute myeloid leukaemia occurring as second malignancy: GIMEMA archive of adult acute leukaemia", *British journal of haematology,* vol. 112, no. 1, pp. 109-117.

Paschka, P., Schlenk, R.F., Gaidzik, V.I., Habdank, M., Kronke, J., Bullinger, L., Spath, D., Kayser, S., Zucknick, M., Gotze, K., Horst, H.A., Germing, U., Dohner, H. & Dohner, K. 2010, "IDH1 and IDH2 mutations are frequent genetic alterations in acute myeloid leukemia and confer adverse prognosis in cytogenetically normal acute myeloid leukemia with NPM1 mutation without FLT3 internal tandem duplication", *Journal of*

clinical oncology : official journal of the American Society of Clinical Oncology, vol. 28, no. 22, pp. 3636-3643.

Pellagatti, A., Jadersten, M., Forsblom, A.M., Cattan, H., Christensson, B., Emanuelsson, E.K., Merup, M., Nilsson, L., Samuelsson, J., Sander, B., Wainscoat, J.S., Boultwood, J. & Hellstrom-Lindberg, E. 2007, "Lenalidomide inhibits the malignant clone and up-regulates the SPARC gene mapping to the commonly deleted region in 5q- syndrome patients", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 104, no. 27, pp. 11406-11411.

Petitjean, A., Achatz, M.I., Borresen-Dale, A.L., Hainaut, P. & Olivier, M. 2007, "TP53 mutations in human cancers: functional selection and impact on cancer prognosis and outcomes", *Oncogene,* vol. 26, no. 15, pp. 2157-2165.

Pichler, M.M., Bodner, C., Fischer, C., Deutsch, A.J., Hiden, K., Beham-Schmid, C., Linkesch, W., Guelly, C., Sill, H. & Wolfler, A. 2011, "Evaluation of mutations in the isocitrate dehydrogenase genes in therapy-related and secondary acute myeloid leukaemia identifies a patient with clonal evolution to IDH2 R172K homozygosity due to uniparental disomy", *British journal of haematology,* vol. 152, no. 5, pp. 669-672.

Rebbeck, T.R., Friebel, T., Lynch, H.T., Neuhausen, S.L., van 't Veer, L., Garber, J.E., Evans, G.R., Narod, S.A., Isaacs, C., Matloff, E., Daly, M.B., Olopade, O.I. & Weber, B.L. 2004, "Bilateral prophylactic mastectomy reduces breast cancer risk in BRCA1 and BRCA2 mutation carriers: the PROSE Study Group", *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* vol. 22, no. 6, pp. 1055-1062.

Rice, K.L., Buzzal, M., Altman, J. & Licht, J.D. 2011, "The molecular basis of acute myeloid leukemia" in *Hematology Education: the education programme for the annual congress of the European Hematology Association* European Hematology Association, , pp. 27-35.

Ripperger, T., Gadzicki, D., Meindl, A. & Schlegelberger, B. 2009, "Breast cancer susceptibility: current knowledge and implications for genetic counselling", *European journal of human genetics : EJHG,* vol. 17, no. 6, pp. 722-731.

Rowe, J.M. & Tallman, M.S. 2010, "How I treat acute myeloid leukemia", *Blood,* vol. 116, no. 17, pp. 3147-3156.

Ruijs, M.W., Verhoef, S., Rookus, M.A., Pruntel, R., van der Hout, A.H., Hogervorst, F.B., Kluijt, I., Sijmons, R.H., Aalfs, C.M., Wagner, A., Ausems, M.G., Hoogerbrugge, N., van Asperen, C.J., Gomez Garcia, E.B., Meijers-Heijboer, H., Ten Kate, L.P., Menko, F.H. & van 't Veer, L.J. 2010, "TP53 germline mutation testing in 180 families suspected of Li-Fraumeni syndrome: mutation detection rate and relative frequency of cancers in different familial phenotypes", *Journal of medical genetics,* vol. 47, no. 6, pp. 421-428.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. & Erlich, H.A. 1988, "Primer-directed enzymatic amplification of DNA with a

thermostable DNA polymerase ", *Science (New York, N.Y.),* vol. 239, no. 4839, pp. 487-491.

Saito, S., Goodarzi, A.A., Higashimoto, Y., Noda, Y., Lees-Miller, S.P., Appella, E. & Anderson, C.W. 2002, "ATM mediates phosphorylation at multiple p53 sites, including Ser(46), in response to ionizing radiation", *The Journal of biological chemistry,* vol. 277, no. 15, pp. 12491-12494.

Sanger, F., Nicklen, S. & Coulson, A.R. 1977, "DNA sequencing with chain-terminating inhibitors ", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 74, no. 12, pp. 5463-5467.

Sauer, M.K. & Andrulis, I.L. 2005, "Identification and characterization of missense alterations in the BRCA1 associated RING domain (BARD1) gene in breast and ovarian cancer ", *Journal of medical genetics,* vol. 42, no. 8, pp. 633-638.

Scardocci, A., Guidi, F., D'Alo', F., Gumiero, D., Fabiani, E., Diruscio, A., Martini, M., Larocca, L.M., Zollino, M., Hohaus, S., Leone, G. & Voso, M.T. 2006, "Reduced BRCA1 expression due to promoter hypermethylation in therapy-related acute myeloid leukaemia", *British journal of cancer,* vol. 95, no. 8, pp. 1108-1113.

Schmid, I., Krall, W.J., Uittenbogaart, C.H., Braun, J. & Giorgi, J.V. 1992, "Dead cell discrimination with 7-amino-actinomycin D in combination with dual color immunofluorescence in single laser flow cytometry ", *Cytometry,* vol. 13, no. 2, pp. 204-208.

Schmitz, B., Radbruch, A., Kummel, T., Wickenhauser, C., Korb, H., Hansmann, M.L., Thiele, J. & Fischer, R. 1994, "Magnetic activated cell sorting (MACS)--a new immunomagnetic method for megakaryocytic cell isolation: comparison of different separation techniques ", *European journal of haematology,* vol. 52, no. 5, pp. 267-275.

Schouten, J.P., McElgunn, C.J., Waaijer, R., Zwijnenburg, D., Diepvens, F. & Pals, G. 2002, "Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification ", *Nucleic acids research,* vol. 30, no. 12, pp. e57.

Seedhouse, C. & Russell, N. 2007, "Advances in the understanding of susceptibility to treatment-related acute myeloid leukaemia", *British journal of haematology,* vol. 137, no. 6, pp. 513-529.

Shen, L., Kantarjian, H., Guo, Y., Lin, E., Shan, J., Huang, X., Berry, D., Ahmed, S., Zhu, W., Pierce, S., Kondo, Y., Oki, Y., Jelinek, J., Saba, H., Estey, E. & Issa, J.P. 2010, "DNA methylation predicts survival and response to therapy in patients with myelodysplastic syndromes", *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* vol. 28, no. 4, pp. 605-613.

Sill, H., Olipitz, W., Zebisch, A., Schulz, E. & Wolfler, A. 2011, "Therapy-related myeloid neoplasms: pathobiology and clinical characteristics", *British journal of pharmacology,* vol. 162, no. 4, pp. 792-805.

Smith, L.M., Sanders, J.Z., Kaiser, R.J., Hughes, P., Dodd, C., Connell, C.R., Heiner, C., Kent, S.B. & Hood, L.E. 1986, "Fluorescence detection in automated DNA sequence analysis ", *Nature,* vol. 321, no. 6071, pp. 674-679.

Smith, R.E., Bryant, J., DeCillis, A. & Anderson, S. 2003a, "Acute myeloid leukemia and myelodysplastic syndrome after doxorubicin-cyclophosphamide adjuvant therapy for operable breast cancer: The national surgical adjuvant breast and bowel project experience", *Journal of Clinical Oncology,* vol. 21, no. 7, pp. 1195-1204.

Smith, S.M., Le Beau, M.M., Huo, D., Karrison, T., Sobecks, R.M., Anastasi, J., Vardiman, J.W., Rowley, J.D. & Larson, R.A. 2003b, "Clinical-cytogenetic associations in 306 patients with therapy-related myelodysplasia and myeloid leukemia: the University of Chicago series", *Blood,* vol. 102, no. 1, pp. 43-52.

Spurdle, A.B., Marquart, L., McGuffog, L., Healey, S., Sinilnikova, O., Wan, F., Chen, X., Beesley, J., Singer, C.F., Dressler, A.C., Gschwantler-Kaulich, D., Blum, J.L., Tung, N., Weitzel, J., Lynch, H., Garber, J., Easton, D.F., Peock, S., Cook, M., Oliver, C.T., Frost, D., Conroy, D., Evans, D.G., Lalloo, F., Eeles, R., Izatt, L., Davidson, R., Chu, C., Eccles, D., Selkirk, C.G., Daly, M., Isaacs, C., Stoppa-Lyonnet, D., Sinilnikova, O.M., Buecher, B., Belotti, M., Mazoyer, S., Barjhoux, L., Verny-Pierre, C., Lasset, C., Dreyfus, H., Pujol, P., Collonge-Rame, M.A., for the GEMO Study Collaborators, Rookus, M.A., Verhoef, S., Kriege, M., Hoogerbrugge, N., Ausems, M.G., van Os, T.A., Wijnen, J., Devilee, P., Meijers-Heijboer, H.E., Blok, M.J., Heikkinen, T., Nevanlinna, H., Jakubowska, A., Lubinski, J., Huzarski, T., Byrski, T., Durocher, F., Couch, F.J., Lindor, N.M., Wang, X., Thomassen, M., Domchek, S., Nathanson, K., Caligo, M., Jernstrom, H., Liljegren, A., Ehrencrona, H., Karlsson, P., for SWE-BRCA, Ganz, P.A., Olopade, O.I., Tomlinson, G., Neuhausen, S., Antoniou, A.C., Chenevix-Trench, G. & Rebbeck, T.R. 2011, "Common Genetic Variation at BARD1 Is Not Associated with Breast Cancer Risk in BRCA1 or BRCA2 Mutation Carriers", *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology,* vol. 20, no. 5, pp. 1032-1038.

Stacey, S.N., Sulem, P., Johannsson, O.T., Helgason, A., Gudmundsson, J., Kostic, J.P., Kristjansson, K., Jonsdottir, T., Sigurdsson, H., Hrafnkelsson, J., Johannsson, J., Sveinsson, T., Myrdal, G., Grimsson, H.N., Bergthorsson, J.T., Amundadottir, L.T., Gulcher, J.R., Thorsteinsdottir, U., Kong, A. & Stefansson, K. 2006, "The BARD1 Cys557Ser variant and breast cancer risk in Iceland", *PLoS medicine,* vol. 3, no. 7, pp. e217.

Starczynowski, D.T., Kuchenbauer, F., Argiropoulos, B., Sung, S., Morin, R., Muranyi, A., Hirst, M., Hogge, D., Marra, M., Wells, R.A., Buckstein, R., Lam, W., Humphries, R.K. & Karsan, A. 2010, "Identification of miR-145 and miR-146a as mediators of the 5q- syndrome phenotype", *Nature medicine,* vol. 16, no. 1, pp. 49-58.

Strachan, T. & Read, A.P. 1999a, "20.1.1 Completion of full-length cDNA sequences can be achieved by assembling overlapping cDNA clones and by RT-PCR based procedures" in *Human Molecular Genetics*, 2nd edn, Bios Scientific Publishers, an imprint of Taylor & Francis Group, New York.

Strachan, T. & Read, A.P. 1999b, "6.1.1 PCR is a cell-free method of DNA cloning" in *Human Molecular Genetics*, 2nd edn, Bios Scientific Publishers, an imprint of Taylor & Francis Group, New York.

Strachan, T. & Read, A.P. 1999c, "6.1.3 The major disadvantages of PCR are the general requirement for prior target sequence information, short size and limiting amounts of product, and infidelity of DNA replication" in *Human Molecular Genetics*, 2nd edn, Bios Scientific Publishers, an imprint of Taylor & Francis Group, New York.

Talwalkar, S.S., Yin, C.C., Naeem, R.C., Hicks, M.J., Strong, L.C. & Abruzzo, L.V. 2010, "Myelodysplastic syndromes arising in patients with germline TP53 mutation and Li-Fraumeni syndrome", *Archives of Pathology & Laboratory Medicine,* vol. 134, no. 7, pp. 1010-1015.

Tefferi, A. & Vardiman, J.W. 2009, "Myelodysplastic syndromes", *The New England journal of medicine,* vol. 361, no. 19, pp. 1872-1885.

Thol, F., Weissinger, E.M., Krauter, J., Wagner, K., Damm, F., Wichmann, M., Gohring, G., Schumann, C., Bug, G., Ottmann, O., Hofmann, W.K., Schlegelberger, B., Ganser, A. & Heuser, M. 2010, "IDH1 mutations in patients with myelodysplastic syndromes are associated with an unfavorable prognosis", *Haematologica,* vol. 95, no. 10, pp. 1668-1674.

Tinat, J., Bougeard, G., Baert-Desurmont, S., Vasseur, S., Martin, C., Bouvignies, E., Caron, O., Bressac-de Paillerets, B., Berthet, P., Dugast, C., Bonaiti-Pellie, C., Stoppa-Lyonnet, D. & Frebourg, T. 2009, "2009 version of the Chompret criteria for Li Fraumeni syndrome", *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* vol. 27, no. 26, pp. e108-9; author reply e110.

Tiu, R.V., Gondek, L.P., O'Keefe, C.L., Elson, P., Huh, J., Mohamedali, A., Kulasekararaj, A., Advani, A.S., Paquette, R., List, A.F., Sekeres, M.A., McDevitt, M.A., Mufti, G.J. & Maciejewski, J.P. 2011, "Prognostic impact of SNP array karyotyping in myelodysplastic syndromes and related myeloid malignancies", *Blood,* vol. 117, no. 17, pp. 4552-4560.

Tiu, R.V., Gondek, L.P., O'Keefe, C.L., Huh, J., Sekeres, M.A., Elson, P., McDevitt, M.A., Wang, X.F., Levis, M.J., Karp, J.E., Advani, A.S. & Maciejewski, J.P. 2009, "New lesions detected by single nucleotide polymorphism array-based chromosomal analysis have important clinical impact in acute myeloid leukemia", *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* vol. 27, no. 31, pp. 5219-5226.

Tutt, A., Robson, M., Garber, J.E., Domchek, S.M., Audeh, M.W., Weitzel, J.N., Friedlander, M., Arun, B., Loman, N., Schmutzler, R.K., Wardley, A., Mitchell, G., Earl, H., Wickens, M. & Carmichael, J. 2010, "Oral poly(ADP-ribose) polymerase inhibitor olaparib in patients with BRCA1 or BRCA2 mutations and advanced breast cancer: a proof-of-concept trial", *Lancet,* vol. 376, no. 9737, pp. 235-244.

Valent, P., Horny, H., Bennett, J.M., Fonatsch, C., Germing, U., Greenberg, P., Haferlach, T., Haase, D., Kolb, H., Krieger, O., Loken, M., van de Loosdrecht, A., Ogata, K., Orfao, A., Pfeilstocker, M., Ruter, B., Sperr, W.R., Stauder, R. & Wells, D.A. 2007, "Definitions and standards in the diagnosis and treatment of the myelodysplastic syndromes: Consensus statements and report from a working conference", *Leukemia research,* vol. 31, no. 6, pp. 727-736.

Vardiman, J.W., Arber, D.A., Brunning, R.D., Larson, R.A., Matutes, E., Baumann, I. & Thiele, J. 2008, "Therapy-related myeloid neoplasms" in *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*, eds. S.H. Swerdlow, E. Campo, N.L. Harris, et al, 4.th edn, IARC, Lyon, pp. 127-129.

Vardiman, J.W. 2010, "The World Health Organization (WHO) classification of tumors of the hematopoietic and lymphoid tissues: an overview with emphasis on the myeloid neoplasms", *Chemico-biological interactions,* vol. 184, no. 1-2, pp. 16-20.

Vardiman, J.W., Thiele, J., Arber, D.A., Brunning, R.D., Borowitz, M.J., Porwit, A., Harris, N.L., Le Beau, M.M., Hellstrom-Lindberg, E., Tefferi, A. & Bloomfield, C.D. 2009, "The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes", *Blood,* vol. 114, no. 5, pp. 937-951.

Varley, J.M. 2003, "Germline TP53 mutations and Li-Fraumeni syndrome", *Human mutation,* vol. 21, no. 3, pp. 313-320.

Ventura, A., Kirsch, D.G., McLaughlin, M.E., Tuveson, D.A., Grimm, J., Lintault, L., Newman, J., Reczek, E.E., Weissleder, R. & Jacks, T. 2007, "Restoration of p53 function leads to tumour regression in vivo", *Nature,* vol. 445, no. 7128, pp. 661-665.

Vojtesek, B., Bartek, J., Midgley, C.A. & Lane, D.P. 1992, "An immunochemical analysis of the human nuclear phosphoprotein p53. New monoclonal antibodies and epitope mapping using recombinant p53 ", *Journal of immunological methods,* vol. 151, no. 1-2, pp. 237-244.

Walter, M.J., Ding, L., Shen, D., Shao, J., Grillot, M., McLellan, M., Fulton, R., Schmidt, H., Kalicki-Veizer, J., O'Laughlin, M., Kandoth, C., Baty, J., Westervelt, P., Dipersio, J.F., Mardis, E.R., Wilson, R.K., Ley, T.J. & Graubert, T.A. 2011, "Recurrent DNMT3A mutations in patients with myelodysplastic syndromes", *Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, U.K,* vol. 25, no. 7, pp. 1153-1158.

Wang, Z. & Chen, Z. 2008, "Acute promyelocytic leukemia: from highly fatal to highly curable", *Blood,* vol. 111, no. 5, pp. 2505-2515.

Whibley, C., Pharoah, P.D. & Hollstein, M. 2009, "P53 Polymorphisms: Cancer Implications", *Nature reviews.Cancer,* vol. 9, no. 2, pp. 95-107.

WijerMans, P., Suciu, S., Baila, L., Platzbecker, U., Giagounidis, A., Selleslag, D., Labar, B., Salih, H., Beeldens, F., Muus, P., de Witte, T. & Lubbert, M. 2008, "Low Dose Decitabine Versus Best Supportive Care in Elderly Patients with Intermediate or High Risk MDS Not Eligible for Intensive Chemotherapy: Final Results of the Randomized Phase III Study (06011) of the EORTC Leukemia and German MDS Study Groups", *ASH Annual Meeting Abstracts,* vol. 112, no. 11, pp. 226.

Yan, X.J., Xu, J., Gu, Z.H., Pan, C.M., Lu, G., Shen, Y., Shi, J.Y., Zhu, Y.M., Tang, L., Zhang, X.W., Liang, W.X., Mi, J.Q., Song, H.D., Li, K.Q., Chen, Z. & Chen, S.J. 2011, "Exome sequencing identifies somatic mutations of DNA methyltransferase gene DNMT3A in acute monocytic leukemia", *Nature genetics,* vol. 43, no. 4, pp. 309-315.

Yin, C.C., Medeiros, L.J. & Bueso-Ramos, C.E. 2010, "Recent advances in the diagnosis and classification of myeloid neoplasms - comments on the 2008 WHO classification", *International journal of laboratory hematology,* .

Zhao, X., Li, C., Paez, J.G., Chin, K., Janne, P.A., Chen, T.H., Girard, L., Minna, J., Christiani, D., Leo, C., Gray, J.W., Sellers, W.R. & Meyerson, M. 2004, "An integrated view of copy number and allelic alterations in the cancer genome using single nucleotide polymorphism arrays", *Cancer research,* vol. 64, no. 9, pp. 3060-3071.

6 Appendix

6.1 Tables

Table 1. WHO classification of myelodysplastic syndromes (MDS) 2

Table 2. Revised FAB classification of acute myeloid leukemia 3

Table 3. WHO classification of acute myeloid leukemia and related neoplasms 4

Table 4. Prognostic groups in AML according to cytogenetics and further subdivision by specific mutations 10

Table 5. The International Prognostic Scoring System (IPSS) 11

Table 6. Classification of chemotherapeutic agents by mechanism of action 15

Table 7. Clinical criteria of LFS and LFL used as a basis for *TP53* germ-line mutation testing 21

Table 8. Under these conditions, chances are over 10% that germ-line mutations in *BRCA1* or *BRCA2* can be found 22

Table 9. Oligonucleotide primer pair used for amplification of exon 7 of the *BARD1* gene 35

Table 10. Oligonucleotide primers for *TP53* exon amplification and direct sequencing 38

Table 11. PCR programs for *TP53* 39

Table 12. Oligonucleotide primers for *PTEN* exon amplification and direct sequencing 40

Table 13. Primary diagnoses of the t-MN patients included in this study 41

Table 14. Primary diagnoses and respective therapy modalities 42

Table 15. Clinical presentation of the t-MN patients 42

Table 16. Bone marrow cytogenetic analysis of the t-MN patients 43

Table 17. Characteristics of index patients tested for germ-line mutations 43

Table 18. Results of *BRCA1* testing 46

Table 19. Results of *BRCA2* testing 47

Table 20. Results of *TP53* testing 48

Table 21. Summary of clinically relevant germ-line mutations found in this study 51

6.2 Figures

Figure 1. Representative illustration of FACS analysis of CD34$^+$ leukemic cells isolated by MACS. Cells show diminished staining for LCA (left plot) and are negative for CD38 (middle plot). The viable cells are depicted as blue events in the right plot. 26

Figure 2. Representative illustration of agarose gel electrophoresis. Exons 1 to 6 of the *PTEN* gene of four different patients were amplified. bp, base pair 30

Figure 3. Capillary electrophoresis and fluorescence detection of oligonucleotide fragments 32

Figure 4. MLPA reaction 33

Figure 5. Representative MPLA ratio chart of patient 6353 tested for BRCA1 large genomic rearrangements (no alteration detected) 34

Figure 6. Heterozygous *BARD1* c.1670G>C germ-line mutation found in fibroblasts of index patient UPN 6387 49

Figure 7. Pedigree of index patient UPN 5824 (arrow). Numbers within symbols indicate the number of unaffected individuals; Bilat, bilateral; BrCa, breast cancer; CIN, cervical intraepithelial neoplasia; LaCa, laryngeal cancer; LuCa, lung cancer; ReCa, renal cancer; y, age in years. 52

Figure 8. Pedigree of index patient UPN 5869 (arrow). Numbers within symbols indicate the number of unaffected individuals; BrCa, breast cancer; GaCa, gastric cancer; Ca, cancer of unknown origin; Leuk, leukemia of unknown type; OvCa, ovarian cancer; y, age in years. 53

Figure 9. Pedigree of index patient UPN 6387 (arrow). BrCa, breast cancer; OvCa, ovarian cancer; UtCa, uterine cancer; y, age in years. 54

Figure 10. Pedigree of index patient UPN 6371 (arrow). BlaCa, bladder cancer; BrCa, breast cancer; Pheo; pheochromocytoma; Plas, plasmacytoma; ProCa, prostate cancer; UtCa, uterine cancer; y, age in years. 55

Figure 11. *BRCA1* c.3112G>T mutation (arrow) in fibroblasts and CD34$^+$ leukemic cells of index patient UPN 5869. 56

Figure 12. *TP53* c.849-852insGGCG mutation in fibroblasts and CD34$^+$ leukemic cells of index patient UPN 6371. 57

Figure 13. *BRCA1* c.5251C>T mutation in fibroblasts and CD34$^+$ leukemic cells of index patient UPN 5824. 58

Figure 14. *TP53* c.1146delA mutation in fibroblasts and CD34$^+$ leukemic cells of index patient UPN 6144. 58

Figure 15. Virtual karyotypes of leukemic cells of index patients UPN 5869 (left two lines) and UPN 6371 (right two lines) show multiple copy number variations. Blue regions indicate gains, red regions losses and violet regions LOH. 59

Figure 16. *BRCA1* locus on chromosome 17q of leukemic cells of index patient UPN 5869 shows copy number state 1 and LOH. Red regions indicate losses and violet regions LOH. 59

Figure 17. *TP53* locus on chromosome 17p of leukemic cells of index patient UPN 6371 shows copy number state 1 and LOH. Red regions indicate losses and violet regions LOH. 60

Figure 18. MSP of genomic DNA from CD34+ leukemic cells (A), breast cancer tumor (B) and fibroblasts (C) of index patient UPN 6144 (c.1146delA) conducted with unmethylated-specific primers shows approximately the same spike pattern representing PCR products and oligonucleotide primers (blue spikes). By contrast, no PCR products could be generated with methylated-specific primers and DNA from $CD34^+$ leukemic cells (D), breast cancer tumor and fibroblasts (not shown), respectively. 61

Figure 19. MSP of genomic DNA from breast cancer tumor (A) and fibroblasts (B) of index patient UPN 6371 shows PCR products and oligonucleotide primers (blue spikes) when conducted with unmethylated-specific primers, but not with methylated-specific primers (C, breast cancer). 62

Figure 20. Immunohistochemical staining of p53 in tumors of index patient UPN 6144. The positive control (A) shows sharp brown staining of lobular carcinoma cells whereas healthy surrounding connective tissue and epithelial cells lining a cyst stay unstained. By contrast, patient´s renal cell carcinoma (B) shows no p53 staining and serous cystadenofibroma (C) indicates a vague staining in some Müllerian epithelial cells (D). 63