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Genomic analysis of an unclassified therapy-resistant abdominal tumour in a child revealed a heterozygous germline mutation in the *BRCA1* gene, in the *RHBDF2* gene and hallmarks of *BRCA*ness

Genomanalyse eines nicht klassifizierten, therapieresistenten pädiatrischen Bauchtumors mit Nachweis einer heterozygoten Keimbahnmutation im *BRCA1*-Gen, im *RHBDF2*-Gen sowie Kennzeichen von *BRCA*ness

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ACTH: Adrenocorticotropic Hormone

ALL: Acute Lymphatic Leukaemia

BRCA1: Breast Cancer Susceptibility Gene 1

BRCA2: Breast Cancer Susceptibility Gene 2

CA 125: Cancer Antigen 125

Chr. : Chromosome

CWS: Cooperative Soft Tissue Sarcoma (“Weichteilsarkom Group“)

DNA: Deoxyribonucleic Acid;

dsDNA: double-stranded DNA

dNTPs: Deoxynucleosidetriphosphates

DSRCT: Desmoplastic Round Cell Tumours

EGFR: Epidermal Growth Factor Receptor

FFT: Fresh Frozen Tissue

GCCR: German Childhood Cancer Register

Gy: Gray unit

HRR Homologous Recombination

HBOC: Hereditary Breast and Ovarian Cancers

ICCC3 Third edition of International Classification of Childhood Cancer

LOH: Loss-Of Heterozygosity”

LOI: Loss Of Imprinting

MAF: Minor Allele Frequency

MRI: Magnetic Resonance Imaging

MIM: Mendelian Inheritance in Man

NaAc: Sodium Acetate

NGS Next-Generation Sequencing SNPs: Single Nucleotide Polymorphisms

NSE: Neuron Specific Enolase

PARP: Poly(ADP-ribose) Polymerase;

PARTNER: Paediatric Rare Tumours Network – European Registry

PCR: Polymerase Chain Reaction

RR: Relative Risk

RB: Retinoblastoma

RHBDF2: Rhomboid 5 Homolog 2 Gen

rpm: rounds per minute

TBE: Tris Base, Boric Acid, EDTA buffer

TSG: Tumour Suppressor Gene

TP53: Transformation-Related Protein 53

WES: Whole-Exome-Sequeincing

WHO: World Health Organisation

Zusammenfassung

Obwohl Krebserkrankungen im Kindesalter als selten gelten (mit weltweit 160.000 Neuerkrankungen/Jahr), stellen sie immer noch die zweithäufigste Haupttodesursache bei Kindern dar.

Die Tumorgenese ist ein hochkomplexer Prozess, an dem häufig somatische Mutationen im Tumor sowie Keimbahnmutationen in Krebsprädispositionsgenen beteiligt sind. Die Untersuchung und das Wissen um mögliche krebsprädisponierende Erkrankungen bei Kindern und Erwachsenen wird zu einem grundlegenden Instrument für die Risikobewertung, Therapieauswahl und Krebsprävention.

Insbesondere bei nicht zu klassifizierenden Tumoren, bei Entitäten mit schlechter Prognose, therapierefraktären Situationen oder gar im Rezidiv, kann die Kenntnis krebsprädisponierender Mutationen zu einem besseren Verständnis der Tumorentstehung führen und den Weg zu gezielten Therapieoptionen eröffnen.

Mit dem Ziel, potenziell umsetzbare genetische Marker mit Optionen für eine personalisierte Therapie zu identifizieren, wurde nach Einholen einer Einverständniserklärung der Sorgeberechtigten das Mutationsprofil eines 11-jährigen Kindes mit rezidivierten, multifokalem nicht klassifiziertem Bauchtumor untersucht. Durch Next-Generation Sequencing (NGS) wurde neben einer heterozygoten somatischen Mutation, eine heterozygote Keimbahn-Missense-Mutation in *BRCA1* (c.3119G>A) und eine heterozygote Keimbahn-Mutation im Rhomboid 5 Homolog 2 *(RHBDF2*) Gen (c.1582G>T) entdeckt (c.737T>C), sowie im Tumorsuppressorgen *TP53* (transformation-related protein 53). Darüber hinaus zeigten die Tumorproben molekulare Merkmale der *BRCAness.*

Obwohl der Prozentsatz der Krebserkrankungen, die im Kindesalter durch zugrundeliegende genetische Mutationen verursacht werden, noch unklar ist, werden in dieser Population zunehmend krebsprädisponierende Erkrankungen festgestellt. Ein Tumorprädispositionssyndrom präsentiert sich in der Regel als eine erkennbare Kombination von phänotypischen Merkmalen, spezifischen Tumorerkrankungen und einer positiven Familienanamnese zur erhöhten Anfälligkeit zur Entwicklung dieser Krebsentitäten. Spezifische Molekulargenetische Alterationen sind nicht immer bekannt. Keimbahnmutationen in *BRCA1* (Breast Cancer Susceptibility Gene 1) oder *BRCA2* (Breast Cancer Susceptibility Gene 2) wurden mit einem

Krebsprädispositionssyndrom im Erwachsenenalter beschrieben. Ihre Prävalenz in pädiatrischen Krebspatienten ist jedoch sehr wahrscheinlich unterschätzt, da eine Mutationsanalyse dieser Gene bei Kindern normalerweise nicht durchgeführt wird.

Die vorliegenden Ergebnisse legen nahe, dass eine umfassende Genomanalyse bei pädiatrischen Tumoren, einschließlich der Gene, die bei Krebsprädispositionssyndromen im Erwachsenenalter beschrieben wurden, nicht nur zu einem besseren Verständnis der Erkrankungsprogression, sondern auch den Weg für individuelle, gezielte Therapieoptionen ebnen kann.

Summary

Although childhood cancer is considered rare (with 160,000 new cases per year worldwide), it is still the second leading cause of child death. Tumourigenesis is a highly complex process that often involves somatic mutations in the tumour and germline mutations in cancer-predisposing genes. Research into and knowledge of possible cancer-predisposing diseases in children and adults is becoming a fundamental tool for risk assessment, therapy selection and cancer prevention.

With the aim of identifying potential actionable genetic markers with options for targeted therapy, the mutational profile of a child with a therapy-resistance and relapsed, multifocal unclassified abdominal tumour was evaluated. Through Next-Generation Sequencing (NGS),a heterozygous germline missense mutation in the tumour suppressor gene *BRCA1* (c.3119G> A) was revealed and a heterozygous germline mutation in the rhomboid 5 homolog 2 *(RHBDF2)* gene (c.1582G > T) was discovered (c.737T> C). Further, a heterozygous somatic mutation (c.737T>C) in the tumour suppressor gene T*P53* (Transformation-Related Protein 53) was detected. The *BRCA1* c.3119G>A mutation has an allele frequency of 1%, and is predicted to be damaging according to Polyphen. A variation in the DNA (Deoxyribonucleic Acid) sequence that occurs in a population with a frequency of 1 % or higher is considered a polymorphism and is generally not considered as pathogenic. Notably, the wild type *BRCA1* allele was significantly reduced throughout the disease progression. In addition, the tumour samples showed molecular characteristics of *BRCAness.*

Although the percentage of childhood cancers caused by underlying genetic mutations is still unclear, increasing cancer predisposing conditions are identified in this population. A tumour predisposition syndrome usually presents as a recognisable combination of phenotypic features, specific tumour diseases, and a positive family history for increased susceptibility to specific cancer subtypes. Germline mutations in *BRCA1* (Breast Cancer susceptibility gene 1) or *BRCA2* (Breast Cancer susceptibility gene 2) have been previously associated with adult-onset-cancer-predisposition syndrome. However, their prevalence in childhood cancer is probably underestimated, as mutational analysis of these genes is not usually performed in this population.

The present findings reveal a potentially underestimated relevance of *BRCA1* in the progression of the child's cancer and address the important and challenging topic of

cancer predisposition genes in the paediatric population. Furthermore, genomic analysis in paediatric tumours, including genes that have been described in adult-onset-cancer-predisposition syndromes, may not only lead to a better understanding of cancer progression, but even pave the way for individualised targeted therapy options.

1 Introduction

1.1 Tumourigenesis

Tumourigenesis is a complex process of cancer development in which genetic and epigenetic changes accumulate over time and in which an initial growth advantage leads to an uncontrolled and invasive proliferation of cancerous cells. Though environmental factors have a principle role in causing sporadic cancer [1], cancer is in essence a genetic disease [2]. The process in which a healthy-cell becomes a tumour-cell differs from cancer to cancer and from individual to individual. The two main properties of cancer cells are uncontrolled cell growth and the ability to invade other tissues. However, there are some recurring mechanism that cause exponential multiplication of unhealthy cells along with a reduced apoptosis. Several factors are implicated in this complex mechanism, such as somatic mutations in the tumour and germline mutations in cancer predisposition genes. Epigenetic alterations can lead to cancer trough DNA methylation (hypermethylation or hypomethylation), Loss Of Imprinting (LOI) and histone modifications [3]. By contrast, genetic alterations include genetic mutations, loss of heterozygosity, genomic instability, and gene copy number variation (CNV). Mutations that affect the tumour genomes range from subtle nucleotide level alterations, including substitutions and small insertions and deletions, to chromosome level alterations, including translocations, inversions, deletions or amplifications. Additionally, whole chromosomes can be lost (aneuploidy) or gained and even the entire set of chromosomes can be multiplied (polyploidy).

Extensive research has been conducted over the last decades to elucidate the role of mutations in the development of certain cancers. So called “driver mutations” can be caused by mutations or epigenetic changes that confer a selective growth advantage to the tumour cells [4]. If the driver mutation occurs in proto-oncogenes or in Tumour Suppressor Genes (TSGs), this generally leads to cancer [5]. Additionally, some genes can act as both proto-oncogenes and TSGs, depending on the context. Proto-oncogenes usually encode for proteins involved in cell growth, cell replication, cell differentiation, and regulation of cell death. When mutated proto-oncogenes turn into oncogenes they have a dominant effect on the cell, accelerating cell division and causing cancer. Further, one mutated allele in these “gain-of-function” mutations is sufficient to cause uncontrolled cell division. Tumour suppressor genes, as their name

suggests, are responsible for slowing down uncontrolled cell division, inducing DNA repair and activating controlled cell death, a process known as apoptosis. Mutations in these genes are recessive at the single cell level, therefore a single mutation is not sufficient to cause carcinogenesis. Tumour suppressor genes can be classified into five categories:

1) Genes encoding intracellular proteins, responsible for the progression into a specific cell cycle stage (e.g. *RB*) [6];

2) Genes encoding signal transducers or receptors for secreted hormones or developmental signals responsible for cell proliferation inhibition (e.g. Adenomatous polyposis coli *APC*) [7];

3) Genes encoding proteins involved in DNA repairing mechanism (e.g. *p53* and DNA mismatch repair protein 2 *MSH2*) [8];

4) Genes encoding checkpoint-control proteins that induce cell cycle arrest in response to DNA damage or chromosomal defects (e.g. *BRCA1*) [9].

5) Genes encoding apoptosis inducing proteins (e.g. *p53)* [7].

While proto-oncogenes have to be “activated”, or turned into oncogenes to cause cancer, tumour suppressor-genes cause cancer even when they are silent. TSG inactivation is common in cancer development and in most heritable cancer forms germline mutations in these genes are causative. “Loss-of-function” mutations in tumour suppressor genes happen to lead to cancer by deactivating an allele in carrier of a germline mutation. With the somatic inactivation of the second allele, “Loss-Of Heterozygosity” (LOH) is induced, as a result the affected individuum ends up having two ineffective alleles of the same gene. In the well accepted Knudson’s “*Two-hit-theory*”, cancer develops when both alleles of a gene are mutated. This can happen on a somatic level, for both alleles, or when in a carrier of a germline mutation, a somatic alteration on the second allele occurs [10]. About 1-5% of common cancers are estimated to be caused by inheritance of a mutated gene. Thus, oncogenes became a major molecular target for anti-cancer targeted therapy.

1.2 Aim of the project

In the case of clear histopathologic and molecular classification of a tumour's entity, standard therapy options including resection, chemotherapy and radiotherapy are usually available within the German Society of Paediatric Oncology and Haematology. This is not the case for rare entities, relapsed tumours or therapy-refractory cancers, as well in cases of high therapy-induced toxicity. Furthermore, when specific, for childhood, uncommon cancer-types develop in very young children, underlying molecular and genetic disposition is suspected, the need to identify driver mutations to customise the patient's therapy is paramount.

With the aim of identifying potential actionable genetic markers to reduce morbidity by tailoring the treatment specifically to the molecular features of the patient's tumour, the mutational profile of a child with relapsed, multifocal unclassified therapy-refractory abdominal tumour was evaluated.

The overall aims of the present work include the categorisation and further analysis of an unclassified, relapsed and therapy-resistant tumour of an 11-year-old child with no further standard therapy option available other than palliative care. Identifying actionable genetic markers increases our ability to understand the following:

- Identification of targetable mutations may open the way to individualised, specific to the patient tailored treatment options.
- Knowledge of specific genetic factors involved in carcinogenesis of sporadic and inherited forms of cancer allows creating personalised prevention strategies, follow up and opens the way to new therapy strategies.

2 Discussion of Literature

2.1 Childhood cancer

Although cancer in childhood is considered rare, (with worldwide 160,000 new cases per year [11]), it still represents the second most common cause of death in children (defined as aged 0-18 years). Childhood malignancies account for around 1% of all cancers. In Germany, the likelihood of a newborn being affected by a malignant neoplasm by the age of 15 is 0.2%. Meaning that one in 450 children is diagnosed with malignant cancer by the age of 15 (German Childhood Cancer Register (GCCR), annual reports 2006 / 07-2009). The causes of childhood cancer development are still largely unknown. To date, various aetiological aspects of exogenous nature such as environmental factors, ionizing and non-ionizing radiation [12] and exposure to pesticides [13], have been discussed. The first description of the association between low doses of ionizing radiation received by the faetus in utero from diagnostic radiography and the subsequent risk of leukaemia and other childhood cancers was demonstrated in the 1950s [14]. In addition to environmental factors, which are of minor importance during childhood, the individual cancer risk is influenced by the individual genetic predisposition to cancer. The fact that most highly aggressive embryonal tumours present at early age and their resemblance to embryonic tissue might be suggestive of the importance of predisposing genetic factors in their development. Recently, inherited genetic mutations have been estimated playing a major role in up to 10% of childhood cancer patients [15].

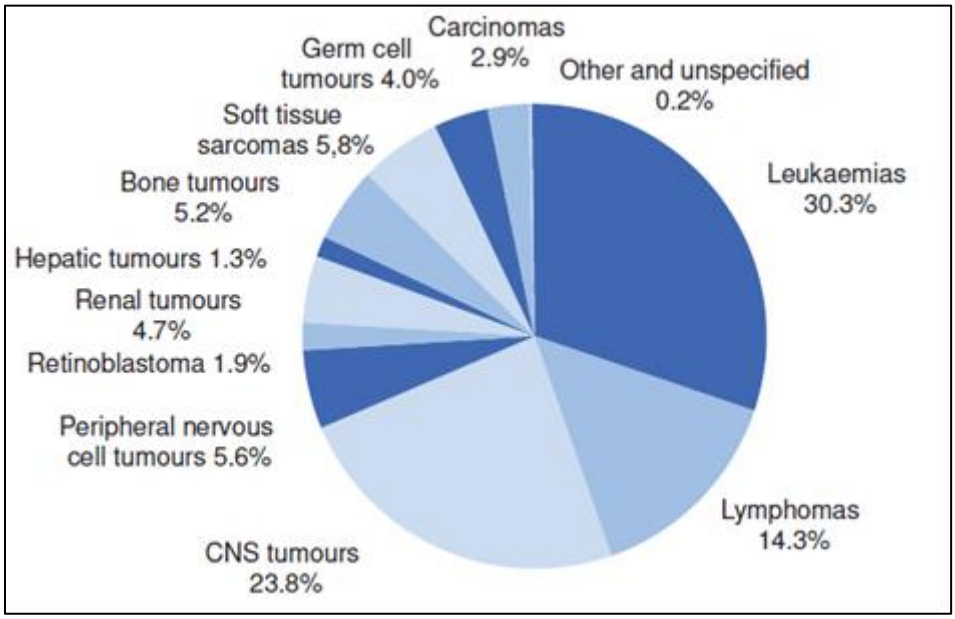
Further, according to the third edition of International Classification of Childhood Cancer (ICCC3), cancer-type distribution is age-specific, and the rate of children being diagnosed with cancer has increased in the last decades [16]. The global cancer incidence in children under the age of 20 has shown that the most frequent entities include leukaemias/myeoloproliferative diseases/myelodysplastic diseases (ICCC group I), followed by brain tumours/miscellaneous intracranial/intraspinal/neoplasms (ICCC group III), lymphomas and reticuloendothelial neoplasms (ICCC group II) [16]. Of the solid tumours, rhabdomyosarcoma, Wilms' tumours (nephroblastoma), neuroblastoma and hepatoblastoma are most frequent. Malignant bone cancers include Ewing sarcomas and osteosarcomas. Further, Steliarova-Foucher et al indicates leukaemia as the most frequent childhood cancer entity in children aged 0-14, followed by central nervous system tumours .

Further, in children aged 15-19 years, lymphomas represent the most common entities followed by epithelial tumours [16]. The distribution of the different entities is similar in Germany where it is reported and analysed annually by the German Childhood Cancer Registry, a population based registry [17]. According to the GCCR, about 2,000 children and adolescents younger than 18 years are annually diagnosed with a malignant disease [18]. Diagnosis, therapy and follow up have improved increasingly in the last decades especially thanks to entity-specific-treatment protocols availability, and therefore a more standardised therapy. In Germany, the treatment of childhood cancer can be considered centralised and offered in the context of trials or based on register suggestions. Paediatric treatment protocols are usually risk-adapted and the multimodal therapy options are adjusted according to the patient's response to treatment with the aim of achieving the greatest long-term survival possible. Even though advances in treatment have increased the overall 5-year survival rate for childhood cancers to approximately 80%, differences in treatment outcome have been described depending on the country the child lives [19]. It has been shown, that in high-income countries, where comprehensive services and healthcare are generally accessible, the likelihood of being cured from cancer is more than 80% [20]. In low- and middle-income countries, only 15-45 % are estimated to be cured from cancer [20]. However, multimodal treatment for childhood cancer is still cost- effective in all income settings.

Despite the fact that most childhood cancers can be successfully cured, there are still rare entities, so called because of their heterogeneity in clinical presentation and of the histopathological classification. A childhood tumour is considered rare in when its annual incidence is <2/million and no standard therapy option or clinical trial are available [11]. These rare entities, when presenting in young age might indicate an underlying genetic cancer predisposition [21]. As a result, once an underlying genetic disorder is suspected, an individualised follow-up program and in some cases, targeted therapy options may be applied with the intention to decrease cancer-related morbidity and finally mortality. For children affected by exceptionally rare tumours, tumour-specific therapy protocols within the German Society of Paediatric Oncology and Haematology (GPOH) are not always available. Nevertheless, patients with rare childhood cancers may benefit from the consultation and advice of the German Paediatric Rare Tumour Group (STEP). This multidisciplinary group of experts on rare paediatric cancers has been active since 2006 and functions as a register since 2012.

STEP provides detailed advice and when possible therapeutic guidelines specifically for rare tumours. Recently, the European (EU) paediatric oncology community developed a three-year EU project that is part of the European Reference Network for Paediatric Cancer (ERN PaedCan), called PARTNER (Paediatric Rare Tumours Network – European Registry). This project aims to create a Paediatric Rare Tumour European Registry dedicated to children and adolescents with very rare tumours by linking existing national registries with the overall goal to improve the care of rare tumours in the paediatric cohort.

Figure 1: Relative frequencies of registered cases reported to the German Childhood Cancer Registry by the main diagnosis groups (2009-2016, based on 16964 patients under 18) [22]



CNS Central nervous system

2.2 Hereditary Cancer Syndromes

Cancer usually occurs in aged individuals but when it occurs in children, it might suggest an increased susceptibility to cancer development. Genetic alterations (mutations) can be inherited and occur as germline mutations or somatic/sporadic mutations [23]. Although the majority of tumours arise sporadically, familial clustering of tumours has been acknowledged since Pierre Paul Broca's first description in 1866 [24]. The surgeon observed the occurrence of breast cancer in 10 members of his wife's family. Hereditary cancer syndromes represent a rapidly expanding field in adult and paediatric haematolgy/oncology. In fact, the percentage of cancer attributed to underlying, inherited genetic mutations has been estimated at up to 10% [15]. Ongoing progress in cancer genetics identifies more predisposing genetic mutations, many of which are often involved in cell-cycle regulation, cell-death pathways and DNA repair systems. The most common cancer predisposition syndromes in paediatric oncology include genetic instability syndromes, DNA damage repair defects, bone failure syndromes, cell cycle and differentiation defects, familial leukaemia syndromes, congenital syndromes and immundeficiencies (Table 1). Despite the enormous expansion in research on genetic susceptibility to childhood cancer, the exact percentage of cancer predisposition in children is probably underestimated. According to a recent study on childhood cancer, germline mutations were found in up to 8.5% of paediatric cancer [15].

Though the study of underlying genetic alterations to cancer is an expanding field, many tumour predisposition syndromes in children are not diagnosed from the beginning of disease-detection as many of them have a low incidence and show variable penetrance. In fact, a syndrome is only present when in the pattern of the anomalies that an individual presents, at least one is of morphologic nature, known or thought to be causal. In some cancer predisposition syndromes, the symptoms or signs leading to the suspition of an underlying syndrome are often age-related due to an age-dependent penetrance. Furthermore, in many paediatric predisposition syndromes, morphological abnormalities can be so subtle that they are easily unnoticed. In many cases, it's not until a specific tumour has already developed that a possible underlying cancer predisposition syndromes is suspected and further analysed. An earlier detection of a cancer predisposition syndrome in the peadiatric cohort might also be

less likely because most paediatricians might not be specifically trained in performing a detailed morphological examination. Moreover, combining seemingly unrelated family history information with the clinical data of the child is not always groundbreaking.

In some cancer predisposition, symptoms aren't present from birth. An example is given by the cancer predisposition syndrome Neurofibromatosis type 1 (NF1), also called Recklinghausen disease, in which the presence of multiple café-au-lait spots in a child often leads the clinician suspecting the underlying cancer predisposition syndrome. The clinical presentation is variable and the diagnosis may not be obvious immediately as café-au-lait spots, axillary and/or inguinal freckling appear usually during the first five years of age and are rarely present from birth. Moreover, cutaneous neurofibromas rarely develop in very young children and are mostly diagnosed after puberty [25]. It is not unusual that the diagnosis of NF1 is performed only after the child developed a CNS-tumour, and not vice versa. However, 15 % of patients with NF1 develop a CNS-tumour, mostly optic nerve astrocytoma and optic nerve gliomas.

Another example in which the search for morphologic anomalies would not necessarily help detecting a cancer predisposition syndrome, is given by the Li-Fraumeni syndrome. Li-Fraumeni syndrome, a cancer predisposition syndrome characterised by the occurrence of multiple tumours in children and in young adults, has been the first fully described cancer predisposition syndrome in childhood [26]. Individuals with Li-Fraumeni syndrome have a 50% lifetime risk of developing cancer before the age of 30 and 90% risk before the age of 60. The predominant cancer entities in this cancer predisposition syndrome are brain tumours, bone and soft tissue sarcomas, adrenocortical and breast cancers. Since its first description, fifty years ago, over 100 genes have been described as being linked to cancer development [15]. In some cancer predisposition syndromes, recognisable syndromic features have been described. Well-established cancer susceptibility syndromes, such as Li-Fraumeni (Mendelian Inheritance in Man MIM 151623), Neurofibromatosis type I (MIM 16220) and Von Hippel-Lindau (MIM 19330), are known to be caused by alterations in specific tumour-suppressor genes (Table 1). Driver mutations in so called "caretaker genes", such as *MLH1* and *MSH2* (encoding for proteins involved in the DNA mismatch repair machinery) and *BRCA1* and *BRCA2* (encoding for proteins in DNA double strand breaks repairs), increase the general mutation rate of the genome, therefore rise

general cancer risk. The study of the subjects' cancer family history, age of presentation as well as the cancer entity may facilitate the presymptomatic identification of high-risk individuals and their relatives who can be screened if appropriate.

Even though in the above mentioned syndromes the clinical presentation was not indicative for a specific cancer predisposition syndrome, it has been recommended that children with an oncologic disease should be assessed either by a clinician geneticist or by a paediatric oncologist with at least genetic training and experience in morphologic anomalies [27]. The first prospective study into the incidence of tumour predisposition syndromes in paediatric patients that has been based on the combination of morphological examination, patients history and family history has been performed by Merks et al. [27]. In their study, 1073 childhood cancer patients underwent a physical examination and in 42 patients (3.9%) a known cancer predisposition syndrome was diagnosed and in another 35 patients (3.3%) a syndrome was suspected, thus diagnostic initiated. However, many syndromes manifest themselves rather as combinations of minor anomalies. Clinical features suspicious of an underlying cancer predisposing syndrome include young onset age, bilateral disease, multiple primary tumours or multifocal presentation, and a positive family history with multiple family members affected by the same tumour type. In conclusion, the knowledge of a specific susceptibility to certain cancer entities might contribute to a better familial understanding and acceptance of the underlying syndrome.

A model example of a cancer predisposition syndrome typically diagnosed on the basis of the child's clinical presentation is given by the Beckwith-Wiedemann Syndrome (BWS) (OMIM 130650) caused by epigenetic and/or genetic alterations in genes on chromosome 11p15.5. Even though the clinical expression is heterogeneous, pathognomonic for this syndrome are overgrowth features, abdominal wall defects, neonatal hypoglycaemia, lateralised overgrowth and the predisposition to embryonal tumours like Wilms tumour, hepatoblastoma or neuroblastoma. For BWS management and surveillance, guidelines are available [28] and considered standard of care for these patients [28].

Another example of a cancer predisposition syndrome in which the clinical presentation of the patient should automatically prompt clinical suspicion of a cancer predisposition is given by Retinoblastoma (RB). RB can be considered the prototype of hereditable cancer predisposition syndromes in childhood [10]. In his pioneering studies, Knudson suggested in 1971 : "*retinoblastoma is a cancer caused by two mutational events. In the dominantly inherited form, one mutation is inherited via the germinal cells and the second occurs in somatic cells. In the non-hereditary form, both mutations occur in somatic cells*" [10]. Since then, Knudsons „*two-hit-theory*" has been used as a guiding principle for many studies on cancers genetic origin. According to this theory, the inherited mutation itself is not sufficient to cause cancer: cells from patients with a cancer predisposition syndrome need to acquire one or more further genetic mutations to develop cancer [10, 29]. The acquirement of a second mutation is not mandatory; therefore, not every carrier of a germline mutation in a cancer susceptibility gene will develop cancer. On the other hand, the occurrence of simultaneous multiple primary tumours has been shown in cancer predisposition syndrome carrier, as the first hit is already present from conception and the second hit can occur independently at different locis. Furthermore, as the first hit mutation is already present from conception, the cancer onset age in the cancer predisposition syndrome carrier is younger compared to their sporadic counterparts in whom cancer is caused by acquired somatic mutations [30].

However, the majority of tumour predisposition syndromes follow an autosomal dominant pattern of inheritance with a recurrence risk of 50% for the following family members. cancer predisposition syndromes can also be inherited in a compound heterozygous pattern or autosomal recessively or X-linked.

Despite the beneficial aspects of testing for underlying genetic predisposition in specific individuals, few ethical considerations should be taken into account, as the tests results may have important psychological and social consequences for the whole family, particularly if the tested individual is a child. Therefore, pre-symptomatic testing should be considered and performed only when a positive test result will have medical consequences [31, 32] and carefully considered and discussed with the parents. If a cancer predisposition syndrome is diagnosed in an individual, a disease-specific lifetime cancer prevention is recommended. In some cases, regular medical visits can induce or trigger anxiety disorders due to the constant fear of developing cancer.

Nevertheless, for unclassified tumours, relapse and therapy-refractory cases, knowledge of cancer predisposing mutations may lead to a better understanding of tumourigenesis and open the way to more targeted therapy options.

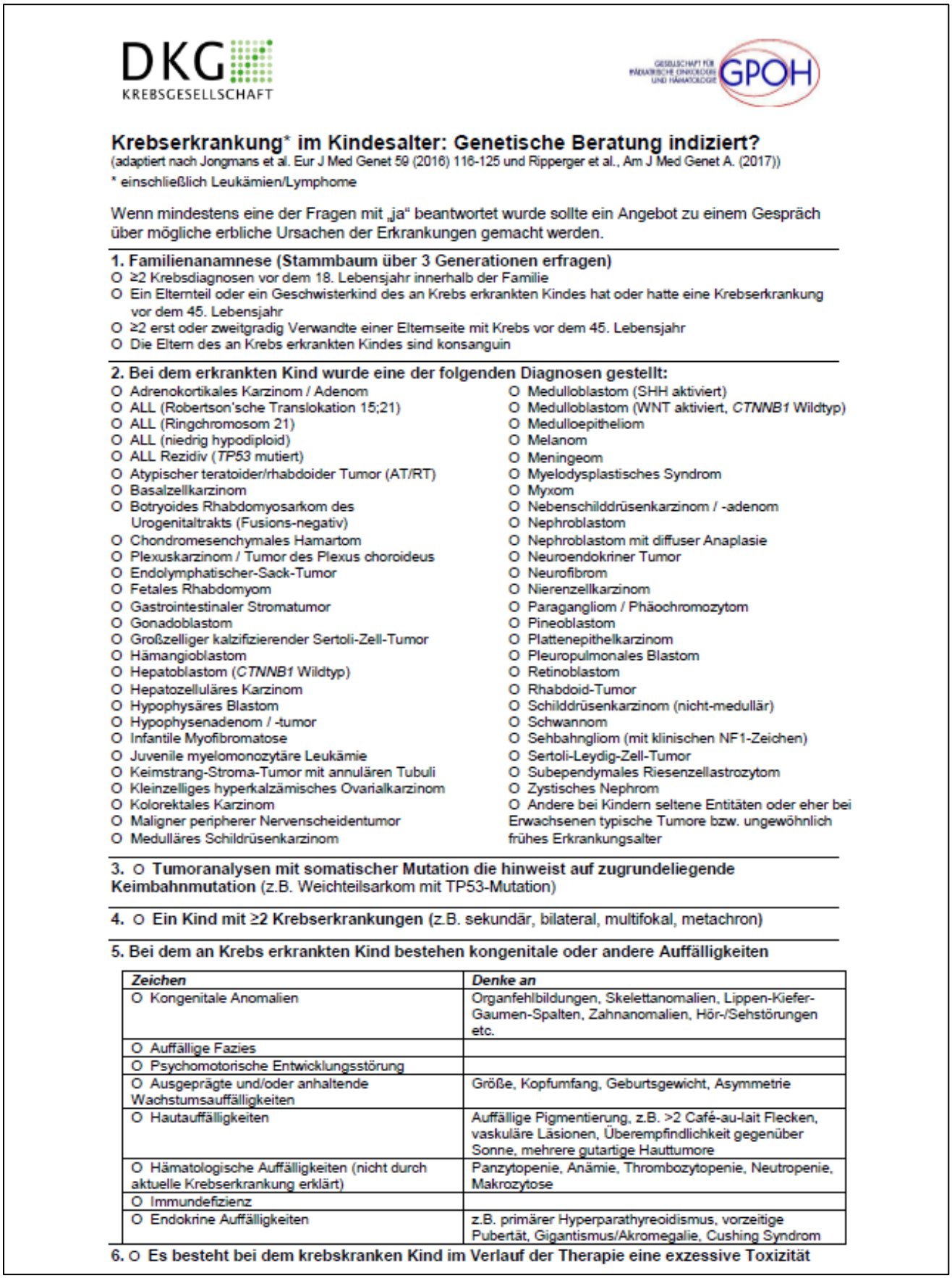
Several recommendations have been proposed for the counselling of genetic tumour predisposition syndromes in children to help and guide professionals in their process of diagnostic advise. Increasing online platforms such as Orphanet (http://www.orphanet.net/consor/cgi-bin/home.php), the National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines in Oncology (http://www.nccn.org/professionals/physician_gls/f_guidelines.asp) and textbooks such as “Management of Genetic Syndromes” by Cassidy and Allanson (are available for professionals with the aim to ameliorate general care of cancer predisposition syndrome carriers.

The Working Group „Genetic Cancer Predisposition“ within the GPOH established a checklist for genetic risk assessment (Figure 2) in 2017. Aiming to facilitate the diagnosis of underlying genetic conditions in the clinical context and the childhood cancer setting, the checklist for genetic risk assessment contains the following items: 1) Family history, 2) Cancer types and/or cancer features known to be associated with a cancer predisposition syndrome, 3) Presence of somatic mutations in the child’s tumour suspicious for underlying germline alterations, 4) Presence of ≥ 2 childhood cancer diagnoses (secondary cancer, bilateral cancer, multifocal cancer, synchronous cancer), 5) Presence of congenital anomalies or other anomalies, 6) Undergone excessive cancer therapy [33].

Although the percentage of childhood cancers caused by underlying genetic mutations is still unclear and probably underestimated, increasing cancer predisposing conditions are identified in this population. Particularly in this cohort of genetically predisposed patients, standard chemotherapy and radiotherapy can cause even more long-term side effects than usual. Further, it can trigger faster progression or secondary malignancies due to therapy-related toxicity. More genome-scale analyses e.g. genotyping, transcriptome sequencing, exome sequencing, and genome sequencing are still needed to identify and elucidate the genetic basis of childhood cancer. Finally,

more screening and multidisciplinary counselling recommendations that take into account the affected and its family are needed.

Figure 2: Checklist for genetic risk assessment



DKG KREBSGESELLSCHAFT

GESELLSCHAFT FÜR PÄDIATRISCHE ONKOLOGIE UND HÄMATOLOGIE GPOH

Krebserkrankung* im Kindesalter: Genetische Beratung indiziert?

(adaptiert nach Jongmans et al. Eur J Med Genet 59 (2016) 116-125 und Ripperger et al., Am J Med Genet A. (2017))

* einschließlich Leukämien/Lymphome

Wenn mindestens eine der Fragen mit „ja" beantwortet wurde sollte ein Angebot zu einem Gespräch über mögliche erbliche Ursachen der Erkrankungen gemacht werden.

1. Familienanamnese (Stammbaum über 3 Generationen erfragen)

- O ≥2 Krebsdiagnosen vor dem 18. Lebensjahr innerhalb der Familie
- O Ein Elternteil oder ein Geschwisterkind des an Krebs erkrankten Kindes hat oder hatte eine Krebserkrankung vor dem 45. Lebensjahr
- O ≥2 erst oder zweitgradig Verwandte einer Elternseite mit Krebs vor dem 45. Lebensjahr
- O Die Eltern des an Krebs erkrankten Kindes sind konsanguin

2. Bei dem erkrankten Kind wurde eine der folgenden Diagnosen gestellt:

- O Adrenokortikales Karzinom / Adenom
- O ALL (Robertson'sche Translokation 15;21)
- O ALL (Ringchromosom 21)
- O ALL (niedrig hypodiploid)
- O ALL Rezidiv (*TP53* mutiert)
- O Atypischer teratoider/rhabdoider Tumor (AT/RT)
- O Basalzellkarzinom
- O Botryoides Rhabdomyosarkom des Urogenitaltrakts (Fusions-negativ)
- O Chondromesenchymales Hamartom
- O Plexuskarzinom / Tumor des Plexus choroideus
- O Endolymphatischer-Sack-Tumor
- O Fetales Rhabdomyom
- O Gastrointestinaler Stromatumor
- O Gonadoblastom
- O Großzelliger kalzifizierender Sertoli-Zell-Tumor
- O Hämangioblastom
- O Hepatoblastom (*CTNNB1* Wildtyp)
- O Hepatozelluläres Karzinom
- O Hypophysäres Blastom
- O Hypophysenadenom / -tumor
- O Infantile Myofibromatose
- O Juvenile myelomonozytäre Leukämie
- O Keimstrang-Stroma-Tumor mit annulären Tubuli
- O Kleinzelliges hyperkalzämisches Ovarialkarzinom
- O Kolorektales Karzinom
- O Maligner peripherer Nervenscheidentumor
- O Medulläres Schildrüsenkarzinom
- O Medulloblastom (SHH aktiviert)
- O Medulloblastom (WNT aktiviert, *CTNNB1* Wildtyp)
- O Medulloepitheliom
- O Melanom
- O Meningeom
- O Myelodysplastisches Syndrom
- O Myxom
- O Nebenschilddrüsenkarzinom / -adenom
- O Nephroblastom
- O Nephroblastom mit diffuser Anaplasie
- O Neuroendokriner Tumor
- O Neurofibrom
- O Nierenzellkarzinom
- O Paragangliom / Phäochromozytom
- O Pineoblastom
- O Plattenepithelkarzinom
- O Pleuropulmonales Blastom
- O Retinoblastom
- O Rhabdoid-Tumor
- O Schilddrüsenkarzinom (nicht-medullär)
- O Schwannom
- O Sehbahngliom (mit klinischen NF1-Zeichen)
- O Sertoli-Leydig-Zell-Tumor
- O Subependymales Riesenzellastrozytom
- O Zystisches Nephrom
- O Andere bei Kindern seltene Entitäten oder eher bei Erwachsenen typische Tumore bzw. ungewöhnlich frühes Erkrankungsalter

3. O Tumoranalysen mit somatischer Mutation die hinweist auf zugrundeliegende Keimbahnmutation (z.B. Weichteilsarkom mit TP53-Mutation)

4. O Ein Kind mit ≥2 Krebserkrankungen (z.B. sekundär, bilateral, multifokal, metachron)

5. Bei dem an Krebs erkrankten Kind bestehen kongenitale oder andere Auffälligkeiten

Zeichen	***Denke an***
O Kongenitale Anomalien	Organfehlbildungen, Skelettanomalien, Lippen-Kiefer-Gaumen-Spalten, Zahnanomalien, Hör-/Sehstörungen etc.
O Auffällige Fazies	
O Psychomotorische Entwicklungsstörung	
O Ausgeprägte und/oder anhaltende Wachstumsauffälligkeiten	Größe, Kopfumfang, Geburtsgewicht, Asymmetrie
O Hautauffälligkeiten	Auffällige Pigmentierung, z.B. >2 Café-au-lait Flecken, vaskuläre Läsionen, Überempfindlichkeit gegenüber Sonne, mehrere gutartige Hauttumore
O Hämatologische Auffälligkeiten (nicht durch aktuelle Krebserkrankung erklärt)	Panzytopenie, Anämie, Thrombozytopenie, Neutropenie, Makrozytose
O Immundefizienz	
O Endokrine Auffälligkeiten	z.B. primärer Hyperparathyreoidismus, vorzeitige Pubertät, Gigantismus/Akromegalie, Cushing Syndrom

6. O Es besteht bei dem krebskranken Kind im Verlauf der Therapie eine exzessive Toxizität

Modified after Rippberger et al [33], German version of the checklist/questionnaire „Kindliche Krebserkrankung – ist eine genetische Beratung indiziert?"[34]

Table 1: Selected common childhood cancer syndromes

Syndrome	Cancer	Genes	MIM
APC-conditions	Colonic polyps, colon cancer, pancreatic cancer, papillary thyroid carcinoma, medulloblastoma, hepatoblastoma, desmoid tumours, osteomas	APC-mutations	175100
Beckwith-Wiedemann Syndrome	Wilms tumour, hepatoblastoma, neuroblastoma, rhabdomyosarcoma	Genetic and epigenetic alterations of chromosome 11p15	130650
Down Syndrome	Childhood ALL, transient myeloid neoplasm, acute megakaryocytic leukaemia	Trisomy 21, GATA1s mutations	190685
DICER1-conditions	Pleuropulmonary blastoma, ovarian sex cord stromal tumours, cystic nephroma, multinodular goiter and tyroid cancer, ciliary body medulloepithelioma, botyroid-type embryonal rhabdomyosarcoma, nasal chondromesenchymal hamartoma, pituitary blastoma, primitive neuroectodermal tumour (PNET), pineoblastoma	DICER1	606241
Fanconi anaemia	ALL, medulloblastoma, hepatocellular carcinoma, nephroblastoma, breast cancer, squamous cell carcinoma	Genes encoding proteins involved in DNA repair	227650
GATA2-deficiency	Myelodysplastic syndromes, higher skin and breast cancer risk	GATA2	137295
Gorlin syndrome	Medulloblastoma, basal cell carcinomas, cardiac and ovarian fibromas, rhabdomyosarcomas, ependymoma, rhabdomyomas	SUFU, PTCH1	109400

Li-Fraumeni syndrome	Soft tissue sarcomas, osteosarcoma, pre-menopausal breast cancer, choriod plexus carcinoma, medulloblastoma, adrenocortical carcinoma, neuroblastoma, leukaemia,	TP53	151623
Neurofibromatosis1	Plexiforme neurofibromas, low-and high-grade gliomas, malignant peropheral nerve sheath tumours, juvenile myelomonocytic leukaemia	NF1	162200
Retinoblastoma	Unilateral and bilateral retinoblastomas, high risk of secondary primary malignancies, e.g., osteosarcoma following radiation, melanoma	RB1	180200
Rhabdoid tumour predisposition 1/2	Renal and extrarenal rhabdoid tumours, meningioma (SMARCB1) and schwannomatosis (SMARCB1), peripheral nerve sheath tumours (SMARCB1), SMARCA4 germline mutations: small-cell carcinoma of the ovary,	SMARCB1, SMARCA4	609322/ 613325

Modified after Rippberger et al [33]

2.2.1 *Breast Cancer susceptibility-gene 1 BRCA1* and *BRCA2* and their occurrence in paediatric cancer

In the literature, the term “susceptibility genes” is used to describe the individual risk of a subject of developing a tumour disease. Germline mutations in *BRCA1* (MIM 604370) or *BRCA2* (MIM 612555) have been associated with adult-onset-cancer-predisposition syndrome due to impairment of the normal protein’s biological activity. The role of *BRCA1* and *BRCA2* in carcinogenesis has been discussed in several reviews and their role as tumour suppressors is accepted [35-37]. Since the first germline pathogenic variant in *BRCA1* has been described in 1994 [38], many more mutations in *BRCA1* and *BRCA2* have been described as the underlying genetic cause of nearly close to 30% of familial breast and ovarian cancer [35, 36, 39]. Germline mutations throughout the *BRCA1* and *BRCA2* genes differ in their penetrance and severity of clinical presentation. Mutations in the *BRCA1* are linked to Hereditary Breast and Ovarian Cancer syndrome (HBOC). Further, one defective germline copy of either *BRCA1* or *BRCA2* is enough to increase the risk of cancer development by 10 to 22 times. The lifetime risk of developing breast cancer by the age of 70 is between 46-87%, depending on the population, the risk for ovarian cancer is 16-44% [40]. Female *BRCA1* mutation carriers have an increased statistical risk of other cancers (Relative Risk RR = 2.30) such as cervical (RR = 3.72), bladder (RR = 2.65) or pancreatic cancer (RR = 2.26). Male carriers over 65 years of age do not have an increased risk of prostate cancer (RR = 0.95), but men under 65 years of age are at risk with a relative risk of RR = 1.82 [41].

BRCA1 and *BRCA2* are not typically sequenced in paediatric cancer patients. So far, a negative family history for tumours that have been already associated to *BRCA* mutations, and missing anomalies in the carrier, may lead to underestimate their prevalence in childhood cancer. However, their role in carcinogenesis and their clinical implications needs to be further studied. To date, the only indication in paediatrics to test for *BRCA1/2* has been in the context of Fanconi anaemia [42, 43] and recently in the context of medulloblastoma [44]. Fanconi anaemia is a rare autosomal recessive or X-linked genetic disease resulting from mutations in one of at least 15 different genes and characterised by several congenital defects, chromosome instability, a predisposition to develop early-onset progressive bone marrow failure, cellular hypersensitivity to DNA crosslinking agents, such as Mitomycin C and Cisplatin. and

an increased risk of developing cancer [45]. At least 4 of these genes (*FANCD1/BRCA2, FANCN/PALB2, FANCJ/BRIP1, FANCO/RAD51C)* are known to be breast/ovarian cancer susceptibility genes. The 15 known genes encode for proteins involved in a common pathway, the so-called Fanconi Anaemia/BRCA pathway, required for the repair of DNA crosslinks [46]. Interestingly a recent study by Domchek et al demonstrates that the BRCA1 protein is also a critical component of this pathway and that *BRCA1* may itself be an FA gene [47].

With increasing NGS profiling, with *BRCA1/2* included in tumour profiling panels, germline mutations in these DNA damage genes have been also detected in paediatric cancers like medulloblastoma [43, 48, 49], neuroblastoma and Ewing sarcoma [49]**.** Waszak and colleagues performed the largest analysis to date on genetic predisposition in a single paediatric brain tumour entity and assessed the actual incidence of cancer predisposition syndromes in patients with medulloblastoma [50]. The group identified six genes with a significant excess of damaging germline mutations in patients with medulloblastoma: *APC, BRCA2, PALB2, PTCH1, SUFU,* and *TP53*. According to this study, the sonic hedgehog subgroup (MB_{SHH}) is characterised by the highest prevalence of genetic predisposition (approximately 20%) with recognised germline driver mutations in *APC*, *BRCA2*, *PALB2*, *PTCH1*, *SUFU* (OMIM *607035), and *TP53*. Remarkably, the study revealed that the cancer predisposition syndrome carriers have a different clinical outcome then non-carrier and the incidence of the specific gene alteration varies among the different medulloblastoma subgroups. For instance, germline mutations in *BRCA2* accounted for the highest proportion among paediatric patients in the medulloblastoma subgroup 3 and 4. Interestingly, all identified *BRCA2* heterozygous germline mutations are rare in the general population (minor allele frequency <0·01%) and were classified in most patients as (likely) pathogenic in ClinVar [50]. Similar to previous studies, the group showed strong signs of genomic instability in heterozygous *BRCA2* mutated medulloblastoma patients. Moreover, the group addresses the need to further evaluate in future studies whether germline carrier with *BRCA2* and *PALB2* mutations and HR-deficient tumours show a particularly favourable response to platinum-based chemotherapy and whether they might benefit from combination-therapies with PARP inhibitors. Therefore, the study and the knowledge of possible cancer-predisposing conditions in the paediatric and adult population is becoming a fundamental tool for

risk assessment, therapy selection and cancer prevention. Even though genetic testing can have a positive impact on the patient's general outcome, it comes along with ethical issues and insecurities on prognosis and quality of life of the affected and it's family [51]. Patients with an underlying genetic predisposition to cancer need also psychological support as well as help in social issues.

Recently a DNA whole-exome sequencing of tumour and normal tissues from 1,120 young patients with different entities of paediatric cancer found germline mutations in 8.5% of the patients [15]. Notably, the second most commonly mutated gene after *TP53* was *BRCA2***.** In this study, a candidate-gene approach has been used; therefore, it is likely that the proportion of paediatric cancer patients harbouring a heritable cancer-susceptibility mutation was underestimated. Accordingly, another recent study estimated that about 7-8% of their paediatric cancer cohort carried a causative predisposing germline variant. Interestingly, also in this study the second mutated gene was *BRCA2 [15]*.

2.2.2 *BRCAness*

The term “BRCAness” has been used to describe a phenotype in which Homologous Recombination Repair (HRR) defects are found in an individual’s tumour in the absence of germline mutations in either *BRCA1* or *BRCA2* [52]. Although there are remarkable clinical, histological and molecular differences between hereditable *BRCA*-mutant cancers and sporadically occurring forms, similar biologic behaviour have been described [53]. Tumours of patients that have features of BRCAness appear to use error-prone DNA-repair pathways resulting in increased genomic instability. This could partially explain their sensitivity to certain DNA damaging agents like platinum salts, mitomycin C and poly-(ADP)-ribose polymerase inhibitors (PARPis) such as olaparib. PARPis induce synthetic lethality by targeting PARP1/2 and blocking the base excision repair mechanism that occurs in the case of HR defects.

The `BRCAness´ phenotype was found to be more pronounced in cancer types with higher genomic instability due to somatic mutations in HRR involved genes such as osteosarcoma. Despite the multimodal treatment approach (surgery, chemotherapy and radiotherapy) for osteosarcoma, known to be one of the most aggressive sarcomas, the overall survival remains poor [54].The molecular approach and the finding that BRCAness is a frequent feature of these tumours, opens the way to the targeted therapy and in specific the use of PARPis. With this aim, Kovac et al. developed two osteosarcoma cell line models and analysed their sensitivity to the PARPis talazoparib [55]. Whether the sensitivity seen in their osteosarcoma cell lines resembles that in *BRCA* gene mutant cell lines remains to be determined.

Further*, BRCA1 and BRCA2* mutations are often accompanied by alterations in *TP53*, which substantially accelerates tumour progression. *TP53* is a well-known tumour-suppressor gene. *TP53* has been described as deleted and mutated in multiple types of tumour independently whether the affected individual is a germline mutation-carrier or the *TP53* alteration occurred somatically. The Cancer Genome Atlas (TCGA) Analysis, published in 2011, indicated alterations in*TP53* in 96% of the analysed HGS-OVCa patients. Further, in the study, at least 50% of the tumour samples analysed, mutations in other HRR involved genes were found. To what extent the interaction between BRCA1/2 mutated proteins and TP53 defect protein might have an accelerating role in carcinogenesis [56] has to be further clarified**.**

With the aim of implementing standard treatments with more drugs targeting specific genetic alterations, the genomic and molecular landscape of BRCAness for the different paediatric cancer entities is being performed by a growing number of centres worldwide. However, to what extent drugs like PARPis will be implemented in paediatric standard treatment trial in combination with other chemotherapeutics or alone remains to be defined.

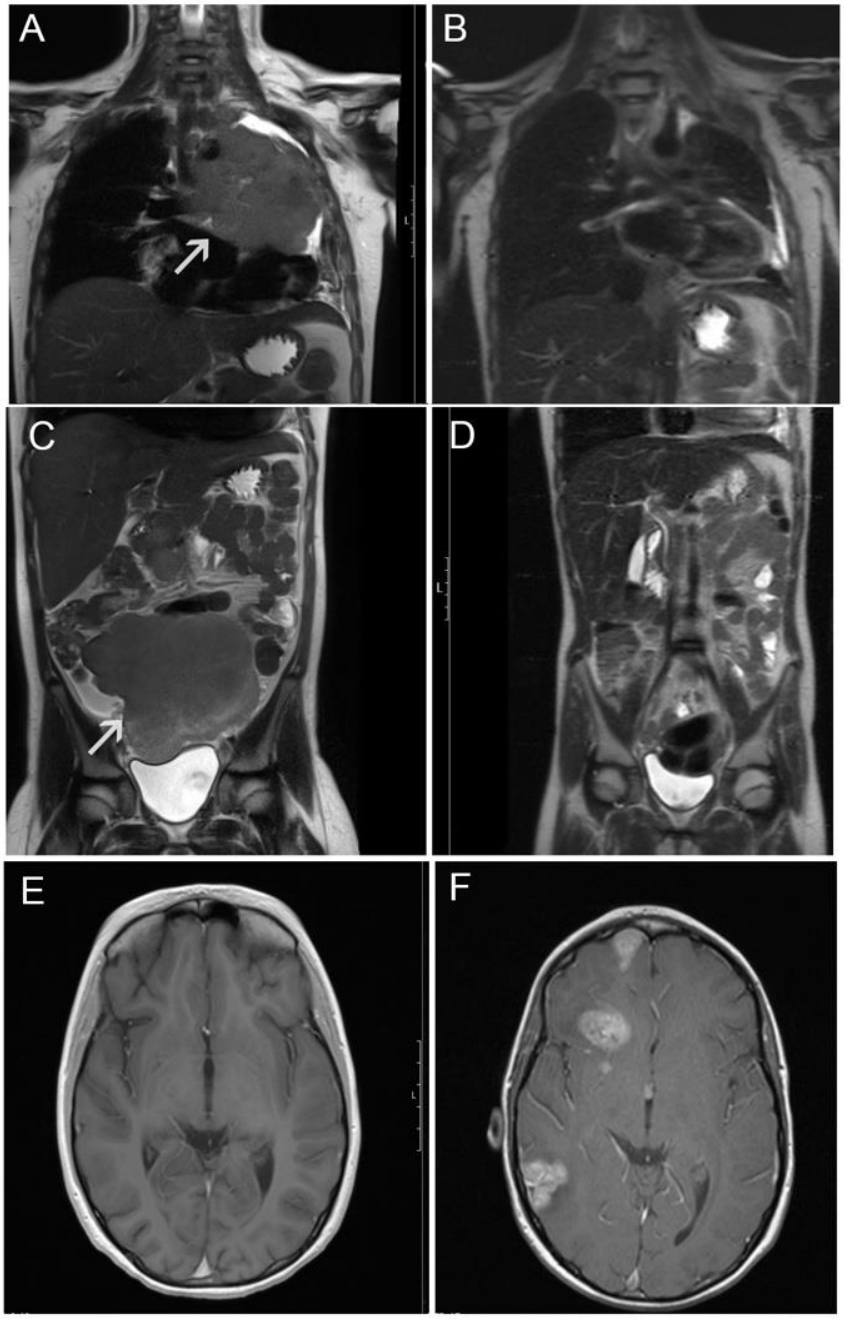
3 Clinical description of the case

3.1 Clinical case

A previously healthy 11-year-old child was referred to our centre with a suspected abdominal mass for further diagnostic evaluation and therapy. The family history was not suggestive for possible hereditary cancer susceptibility. The patient presented a 6-week history of worsening abdominal pain, culminating in severe obstipation and hyperemesis. Furthermore, history of coughing with progressive shortness of breath during the previous eight months was provided. Clinical examination showed a distended abdomen and a palpable solid mass, the auscultation of the chest revealed a silent left lung. The further clinical evaluation did not reveal any other abnormalities, specifically no morphological abnormalities were found. Until this date, medical history, neurological development was unexceptional and the patient's growth chart indicated normal development. Whole-body magnetic resonance imaging (MRI) revealed multiple abdominal (largest mass: 13.3 x 9.4 x 11.7 cm, Figure 3) and thoracic tumour masses (largest mass: 10.5 x 10.8 x 11.5 cm, Figure 3) with pleural and pericardial effusions (Figure 3). Blood tests showed increased levels of Neuron Specific Enolase (NSE), Cancer Antigen 125 (CA 125) and Adrenocorticotropic Hormone (ACTH). The child revealed features of pulmonary artery hypertension due to combination of bilateral pulmonary vein stenosis along with bilateral stenosis of the pulmonary arteries, most likely through compression by the left thoracic tumour masses resulting in left lung atelectasis. Echocardiography and chest radiography revealed an increased cardiac silhouette through pericardial effusion, as well as a pleural effusion resulting from tumour spread.

3.2 **Imaging**

Figure 3: Magnetic resonance imaging of the child's tumours

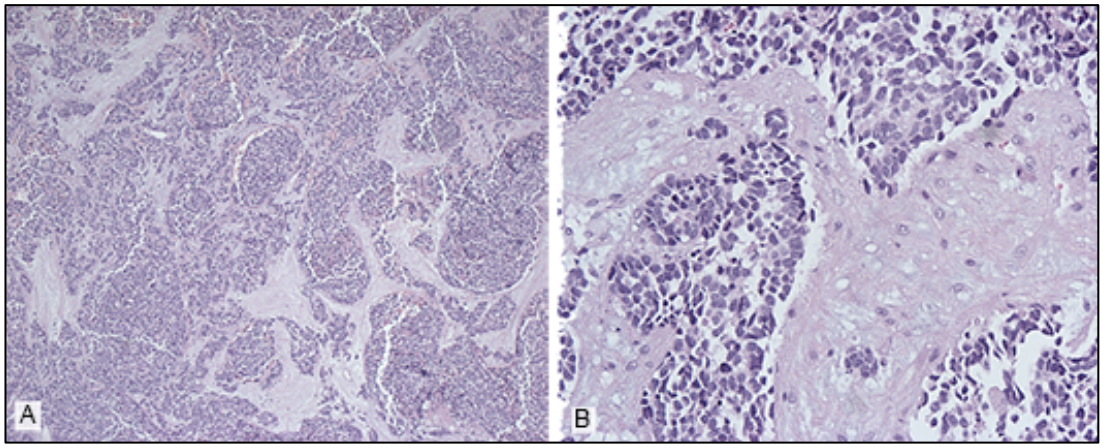


MRI of the thorax [A] before and [B] after chemotherapy; MRI of the abdomen before [C] and after chemotherapy [D]; MRI of the brain at diagnosis [E] and with metastasis [F]. Figures kindly provided by Univ.-Prof. Dr. G. Staatz (Section of Pediatric Radiology, Department of Diagnostic and Interventional Radiology, University Medical Center of the Johannes Gutenberg-University Mainz).

3.3 **Pathologic examination**

The biopsy of the biggest abdominal tumour mass (sample no 109) was characterized predominantly by nests of undifferentiated small neoplastic cells within abundant desmoplastic stroma (Figure 4). The histological, immunohistochemical and cytogenetic features of the child's tumours were not specific to any known paediatric cancer entity. The histopathologic criteria (stroma-rich small round blue-cell tumour) and immunohistochemical positivity for cytokeratin (CK-MNF, EMA, CK7) and neuroendocrine markers (neuron-specific enolase / chromogranin / synaptophysin) of all tumour samples of the child were consistent with the diagnosis of desmoplastic round cell tumours (DSRCT). Desmoplastic round cell tumours are considered rare malignant tumours of the soft tissue sarcoma family, usually arising from the abdomen and pelvic organs. DSRCTs are associated with very poor prognosis. However, desmin staining was negative and no characteristic chromosomal translocation of t (11; 12)(p13;q12) between Ewing's sarcoma (EWS) gene on chromosome 22 and Wilm's tumour gene on chromosome 11, leading to an EWS-WT1 fusion, pathognomonic for DSRCT, was found. The histopathologic criteria and immunohistochemical positivity for CD56 and reactivity for neuron-specific enolase / chromogranin / synaptophysin was demonstrated in all tumour samples, supporting differential diagnosis of neuroectodermal malignancies but no characteristic translocations of the Ewing sarcoma family (EWSR1 /WT1/ FUS/CIC gene) were found in the patient. Although the histological presentation was similar to soft tissue sarcoma, distinction from other small round-cell tumours, Ewing sarcoma and neuroectodermal tumours, normally based on their immunophenotypic pattern, was not possible. Despite the multifocal presentation at diagnosis (thoracic, pericardial and abdominal), the presumed origin was the right ovary.

Figure 4: Histopathologic features of the primary tumour



Abdominal DSRCT-like tumour. Undifferentiated tumour cells were arranged as nest bulk, surrounded by a dense desmoplastic fibrous stroma (hematoxylin-eosin staining; original magnification x100 [A] and x200 [B]. Figures kindly provided by Dr. L. Seidmann, Institute of Pathology, University Medical Center of the Johannes Gutenberg-University Mainz.

3.4 **Therapy**

Neoadjuvant high dose chemotherapy was initiated according to the German risk adapted soft tissue sarcoma therapy-guideline “CWS-Guidance” and included carboplatin, etoposide, vincristine, actinomycin D, ifosfamide, and doxorubicine. After two courses of chemotherapy, a partial response was achieved (**Figure 3**). Subsequently, after four courses of chemotherapy, abdominal and thoracic surgical tumour-debulking with right ovariectomy was performed. According to the treatment protocol, after the surgery, radiotherapy of the left hemithorax with in total 46 Gray unit Gy was performed in parallel to further five courses of chemotherapy. Treatment was considered ended after nine courses of standardised chemotherapy, after which regular follow-up was performed. In the whole-body imaging obtained two months after completion of radiochemotherapy, metastatic disease was revealed. The Magnetic Resonance Imaging (MRI) of the brain showed multiple cerebral masses with diffuse contrast enhancement consistent with metastases (Figure 3). The lumbar punction confirmed the metastatic disease by the presence of tumour cells in the cerebrospinal fluid. Furthermore, the recurrence of left thoracic mass, as well as new intraabdominal and pelvic tumour-lesions were detected. Histopathologic evaluation of the brain metastasis revealed the same morphological and immunohistochemical patterns as the primary tumour. Palliative craniospinal irradiation (30 Gy) was initiated. At this point the option of targeted therapy with PARP-inhibitors was discussed, but due to further massive tumour progression and sudden death not applicable .

4 Material and Methods

4.1 Ethical considerations

This study was performed after informed consent was obtained from the patients' legal guardian. The mutational analysis of the patient's tumour samples were performed retrospectively on stored surplus material, after standard therapy failed with the intent to find targetable mutations. Germline analysis based on the suspicion of *BRCA1/BRCA2* was performed with the same intentions. This study was performed in agreement with the declaration of Helsinki on the use of human material for research.

The ethical aspects of genetic counseling in the paediatric cancer cohort was discussed in chapter 2.2. and in the final discussion of this thesis.

Material

4.2 Collection of the patient's biomaterial

Peripheral blood samples were collected during routine biomaterial collections at diagnosis, and during therapy monitoring. After informed consent was obtained, ssurplus biomaterial was initially stored. Tumour samples were routinely collected for histopathologic diagnosis from the patient after informed consent from the patient's mother was obtained. For this study, Fresh Frozen tumour samples were available from the primary tumour at the time of the first surgery and from a brain metastasis at the time of metastatic disease. Tumour samples were analysed by a pathologist of the university centre, regions containing vital tumour cells were isolated for further processing.

4.2.1 Tools and related equipment

Table 2: Utilised tools and related equipment

Tools and related equipment	Labeling	Manufacturer
Autoclave		INTEGRA Biosciences GmbH, Fernwald
Bunsen burner	Blue Flame GB2001 mini bunsen burner	Blazer, unknown
Centrifuge	Heraeus Sepatech Megafuge 1.0	Thermo Fisher Scientific, Waltham, MA, USA
Centrifuge	Heraeus Megafuge 8R	Thermo Fisher Scientific, Waltham, MA, USA
Centrifuge	VWR MicroStar 12	VWR science education, Radnor, PA, USA
Centrifuge	awel centrifugation C20-R	Alliance Bio Expertise (A.B.E), Blain, France
Centrifuge	Eppendorf centrifuge 5430 R	Eppendorf AG, Hamburg
Centrifuge	Eppendorf centrifuge 5417 R	Eppendorf AG, Hamburg
Computer	Optiplex 7020	Dell Inc., Round Rock, TX, USA
Electrophoresis system	Consort 300 V/1000 mA E831	VWR science education, Radnor, PA, USA
Fridge-freezer 4°C/-20°C	Liebherr comfort	Liebherr-International AG, Bulle, Switzerland
Gel documentation system		Nippon Genetics EUROPE GmBH, Düren
Heating block	ThermoQ	Biozym Scientific GmbH, Hessisch Oldendorf
Sequencer	MiSeq	Illumina
Microwave	unknown	unknown
Nitrogen tank	MVE CryoSystem 4000	Chart Industries Inc., Mineral, VA, USA
PCR cycler	Mastercycler gradient	Eppendorf AG, Hamburg

PCR cycler	Mastercycler nexus gradient	Eppendorf AG, Hamburg
Pipettes	Eppendorf Research® plus, 0,1-2,5 µl, 0,5-10 µl, 2-20 µl, 10-100 µl, 100-1000 µl	Eppendorf AG, Hamburg
Pipettes	Eppendorf Reference® 2, 2-20 µl	Eppendorf AG, Hamburg
Pipettes	Finnpipette F1, F2, F3	Thermo Fisher Scientific, Waltham, MA, USA
Pipetting aid	Pipetus®	Hirschmann Laborgeräte GmbH & Co. KG, Eberstadt
Pipetting aid	Integra Vacuboy	INTEGRA Biosciences GmbH, Fernwald
qRT-PCR cycler	LightCycler® 480 II	Roche Diagnostics International AG, Rotkreuz, Switzerland
Rocking shaker	Gallenkamp orbital shaker incubator	Thermo Fisher Scientific, Waltham, MA, USA
Safety workbench	Comfort Holten Flow Master	Thermo Fisher Scientific, Waltham, MA, USA
Safety workbench	Holten Lamin Air 1,2	Thermo Fisher Scientific, Waltham, MA, USA
Sequencer	ABI Prism 3130 Genetic Analyzer	HITACHI, Thermo Fisher Scientific, Waltham, MA, USA
Spectrophotometer	Nanodrop 2000	Thermo Fisher Scientific, Waltham, MA, USA
Table scale	GIBERTINI Europe 1000	Gibertini Elettronica S.r.l, Novate Milanese, Italy
Tissue lyser	Tissue Lyser II	Qiagen GmbH, Hilden
Ultra-low freezer -80°C	HERA Freeze HFU B Series	Thermo Fisher Scientific, Waltham, MA, USA
Vortex	IKA® MS3 basic	IKA®-Werke GmbH & Co. KG, Staufen
Water bath	Julabo 17	JULABO GmbH, Seelbach
Water bath	DC 10	Thermo Fisher Scientific, Waltham, MA, USA

4.2.2 Chemicals

Table 3: Utilised chemicals

Chemicals	Labeling	Manufacturer
Agar		AppliChem GmbH, Darmstadt
Ammonium chloride	NH_4CL, powder	Merck KgaA, Darmstadt
D-PBS	Dulbecco´s phosphate buffered saline	Sigma Aldrich Corp., St. Louis, MO, USA
Ethanol	70%, 100%	Honeywell International, Morristown, NJ, USA
Ethylenediaminetetraacetic acid	EDTA	Sigma Aldrich Corp., St. Louis, MO, USA
Formamide	Highly-deionized (Hi-Di) formamide	Life technologies, Carlsbad, CA, USA
Orange G		Sigma Aldrich Corp., St. Louis, MO, USA
Potassium bicarbonate	$KHCO_3$, powder	AppliChem GmbH, Darmstadt
Sodium acetate		Sigma Aldrich Corp., St. Louis, MO, USA
Sodium chloride		Carl Roth GmbH & Co. KG, Karlsruhe
Tris(hydroxymethyl)aminomethane	TRIS	Carl Roth GmbH & Co. KG, Karlsruhe
Water	H_2O, HPLC grade	Sigma Aldrich Corp., St. Louis, MO, USA

4.2.3 Consumables

Table 4: Utilised consumables

Consumables	Labeling	Manufacturer
96-well plates	Corning™ Costar™ Flat Bottom Cell Culture Plates with lid, PS, sterile	Thermo Fisher Scientific, Waltham, MA, USA
10x reaction buffer		Axon Labortechnik GmbH, Kaiserslautern
Betaine		Sigma Aldrich Corp., St. Louis, MO, USA
Deoxynucleosidetriphosphates	dATP, dCTP, dGTP, dTTP	Promega GmbH, Mannheim
Disinfection solution	Terralin® Liquid	Schülke & Mayr GmbH, Nordernstedt
Magnesium chloride	MgCl	Axon Labortechnik GmbH, Kaiserslautern
Midori Green		Nippon Genetics EUROPE GmbH, Düren
PCR plates, 96-well	LightCycler® 480 Multiwell Plate 96, white	Roche Diagnostics GmbH, Mannheim
PCR tubes	0,2 ml thin-walled tubes with flat caps	Thermo Fisher Scientific, Waltham, MA, USA
Pipette tips 10 µl, 1250 µl	Safe Seal SurPhob tips, sterile, no filter	Biozym Scientific GmbH, Hessisch Oldendorf
Pipette tips 100 µl, 200 µl	TipOne®, filter	STARLAB GmbH, Hamburg
Reaction tubes 1,5 ml, 2 ml	Safe-Lock Tube 1,5 ml, 2 ml	Eppendorf AG, Hamburg
SYBR Green Mastermix	KAPA SYBR FAST MasterMix	VWR science education, Radnor, PA, USA

Tubes 15 ml	CELLSTAR® Tubes, 15 ml, PP, graduated, conical bottom, blue screw cap, sterile	Greiner Bio One, Frickenhausen
Tubes 50 ml	CELLSTAR® Tubes, 50 ml, PP, graduated, conical bottom, blue screw cap, sterile	Greiner Bio One, Frickenhausen
Tubes 5 ml	5 ml PP tube, round bottom, sterile	Greiner Bio One, Frickenhausen
Tubes 14 ml	BD Falcon Round-Bottom Tube, PP, 14 ml	Becton Dickinson Bioscience, Heidelberg
Taq-Polymerase	Taq-Polymerase 5U/µl	Axon Labortechnik GmbH, Kaiserslautern

4.2.4 Kits

Table 5: Utilised kits

Kit	Labeling	Manufacturer
DNA extraction	Fast DNA Tissue Kit	
cDNA synthesis	TaKaRa Prime Script RT reagent Kit with gDNA Eraser	Takara Bio Europe, Saint-Germain-en-Laye, France
Sequencing	BigDye v3 Terminator Kit	Thermo Fisher Scientific, Waltham, MA, USA
Sequencing	TruSight	Illumina

4.2.5 Primers

Primers for Sanger Sequencing

Table 6: Utilised primers for Sanger Sequencing

Primer	Sequence
***BRCA1* forward**	*5'- AGCATTCAATTTTGGCCCTCTG*
***BRCA1* reverse**	*5'- CACCACTTTTTCCCATCAAGTCA*
***TP53* forward**	*5'- GGCGCACTGGCCTCATCT*
***TP53* reverse**	*5'- GGGGTCAGAGGCAAGCAG*
***RHBDF2* forward**	*5'- GTCCTTGCCCACTCCAGT*
***RHBDF2* reverse**	*5'- GTGGGTGGCTCAGATGGCA*

4.2.6 Size standard

Table 7: Utilised size standards

Size standard	Manufacturer
100 bp DNA ladder	New England Biolabs GmbH, Frankfurt

4.2.7 Software

Table 8: Utilised software

Software	Manufacturer
LightCycler® 480 Software	Roche Diagnostics International AG, Rotkreuz, Switzerland
Microsoft Office	Microsoft, Redmond, WA, USA
Sequencher 5.0	Gene Codes Co., Ann Arbor, MI, USA

Methods

4.3 Nucleic acid extraction

The tumour samples were analysed by a pathologist of the university medical centre and regions containing vital tumour cells were isolated for further processing.

4.3.1 DNA Extraction from Fresh Frozen Tissue (FFT)

The DNA extraction from FFT was performed using QIAamp Fast DNA Tissue Kit (Qiagen) following the manufacturer's instructions, which is specified to allow up to 40pg of DNA to be extracted from 25mg of soft tissue. The manufacturer's protocol was followed. Firstly, the tissue was previously analysed and selected by a pathologist, only tissue containing vital cancer cells were cut into small pieces. Secondly lysed, precipitated with ethanol and added to a spin column to which the DNA bound. After several washes, the DNA was eluted from the column with distilled water.

4.3.2 Extraction of genomic DNA

The extraction of genomic DNA from peripheral blood was performed by standard methods from peripheral blood using Puregene® Blood Core Kit B from Qiagen, according to the manufacturer's instructions, or using the ammonium acetate method set out as follows. The first steps of this method acted to break down the cell wall to allow access to the nucleus. Nine ml frozen blood samples were thawed and the blood transferred to a 50ml Falcon tube. Ice cold water was added to the tubes to give a final volume of 50mls, and then the tube inverted to mix and lyse the red blood cells. The tubes were then centrifuged at 2300 rpm for 25 minutes at 4°C in a swing out rotor centrifuge. The supernatant was discarded by inverting the tube gently, being careful not to disturb the pellet. The tube was inverted and placed on a clean paper towel to remove the last traces of supernatant. The pellet was then washed with 25 ml 0.1% NP-40 (Sigma) and centrifuged at 2300rpm for 20 minutes at 4°C. The wash was repeated if necessary. The supernatant was discarded and the tube inverted over a paper towel. To lyse the nuclei, 3ml nuclei lysis buffer was added to the pellets and the tube vortexed to resuspend the pellet completely. 200pl 10% SDS and 600 pl proteinase K solution was then added to the tube to degrade any protein. The solutions were mixed by inversion and incubated at 60°C for 11/2-2 hours or overnight at 37°C. Following proteinase K digestion, 1 ml of saturated ammonium acetate solution (148g

NH4AC (BDH) in 50mls distilled water) was added and the tube vortexed vigorously for 15 seconds. The tube was left to stand at room temperature for 20 minutes, and then centrifuged at 2300 rpm for 20mins at room temperature. The DNA was then present? in the supernatant and ready to be precipitated. The supernatant was transferred to a clean tube 50-ml falcon tube, and two volumes of ice-cold ethanol added. The contents were mixed by gentle inversion, and then the DNA was spooled out using either a fine glass rod or a fine plastic sterile loop. The spooled DNA was dipped into an Eppendorf tube® containing 70% ethanol (to wash the DNA and to remove any salts). The DNA was transferred to a labelled screw capped Eppendorf tube®, left to dry and then re-suspended in 1ml distilled water. To assess quantity and quality, an aliquot was diluted 1:50 and analysed by spectrophotometry at 260 and 280 nm.

4.4 Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) is the applied method to amplify certain segments of the DNA. This method is used to amplify regions of target DNA. Portions of the sequence which flank the desired target are used to design two synthetic DNA oligonucleotides, one complementary to each strand of the DNA double helix. These oligonucleotides serve as primers for in vitro DNA synthesis, which is catalysed by a thermostable DNA polymerase, with the primers determining the ends of the amplified DNA fragment.

The method can be succinctly divided into three steps – denaturation, hybridisation and elongation First, the template DNA strands have to be separated into two single strands by denaturation at 94°C for 5 minutes, then 30-35 cycles each of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds. Afterwards, hybridisation of the specific forward and reverse primers and the single-stranded template DNA is induced by reducing the temperature to the optimum for the inserted primers. A final extension step in which temperature is increased to 72°C for 10 minutes ensures the optimal conditions for the Taq-Polymerase that elongates the primer to reproduce a double-stranded DNA. As the reaction proceeds simultaneously at both template DNA strands, the amount of template DNA is doubled per cycle.

The reaction solution (per sample) applied in this work is shown in the following:

10.5 µl of H_2O

2.5 µl of buffer

5 µl of betaine

1.5 µl of MgCl

1 µl of forward primer (1:10 dilution)

1 µl of reverse primer (1:10 dilution)

2 µl of deoxynucleosidetriphosphates (dNTPs)

0.5 µl of Taq-Polymerase I (Axon Labortechnik)

1 µl of template cDNA

The samples were incubated under the conditions shown in the table below:

Table 9: PCR program

Steps	Temperature [°C]	Time [s]	cycles
Initial Denaturation	94	300	1
Denaturation	94	30	
Annealing	60	30	40
Extension	72	60	
Final Extension	72	600	1

Afterwards, PCRs were visualised in agarose gels as described in 4.6.

4.5 Agarose gel electrophoresis

To visualise and verify PCR results, agarose gel electrophoresis was performed. This method is based on the separation of macromolecules such as DNA or proteins in an agarose matrix by application of an electric field moving the charged molecules through the matrix. Smaller molecules travel faster than larger molecules.

In this work, agarose gel electrophoresis was used to separate DNA fragments. The moving rate of double-stranded DNA (dsDNA) is inversely proportional to the logarithm of the number of base pairs - by length to identify the different isoforms amplified in a PCR before. Agarose was prepared by boiling a mixture of agarose powder (Gibco BRL) in 1xTBE. Here, 1% horizontal agarose gels were prepared. 1.8 g of agarose powder were solubilised in 130 ml of 1xTBE buffer by microwave heating and 6 µl of Midori Green (Nippon Genetics) were added for staining. Midori Green is a dye that intercalates with DNA or RNA and fluoresces under UV light, allowing visualisation of the DNA fragments. Molten agarose was poured into a gel-casting tray with a comb in position and left to set. The comb was then removed; the gel was placed in a running tank and then covered with a running buffer of 1xTBE. Wells were loaded with each 6 µl of each sample and 6 µl of a 100 bp DNA ladder (New England Biolabs) as size standard. The gels were run at a maximal voltage of 130 V for 45 minutes and visualised under UV light by a gel documentation system by Nippon Genetics Europe.

4.6 Genetic analysis

4.6.1 NGS Analysis

To determine the nucleotide sequence of the DNA of 94 target genes and 284 SNPs previously identified to be involved in cancer predisposition and oncogenesis (TruSight Cancer panel, Illumina, FC-121-0202) we performed Sanger sequencing. 50 ng DNA of sample 109 was used for the library construction according to the manufacturer's instructions (Nextera Rapid Capture). Paired-end sequencing was performed on an Illumina MiSeq platform (2x150 cycles). Data were processed using BWA Enrichment v1.0 for the generation of BAM files and GATK for variant calling. Analysis of variants was performed with the VariantStudio software (Illumina).

Sanger Sequencing

DNA sequencing was performed after amplification of the region of intertest by PCR. The steps are described in the following paragraphs .

4.6.2 PCR purification

PCR products were purified by an enzymatic method to remove leftover primers as well as remaining dNTPs. Enzymes used were 10 IU exonuclease I and 2 IU shrimp alkaline phosphatase (SAP) (New England Biolabs). The master mix added to each PCR product consisted of the following components:

0.2 μl of exonuclease I

0.8 μl of SAP

3 μl of HPLC H_2O

Samples were incubated under following conditions afterwards:

Table 10: Program for thermal cycler for PCR purification

Steps	Temperature [°C]	Time [min]	cycles
Enzyme incubation	37	30	1
Enzyme activation	80	15	1
Cooling	16	∞	1

4.6.3 Sequencing reaction

The method of Sanger Sequencing is based upon the enzymatic incorporation of dideoxyribonucleoside triphosphates in which the deoxyribose 3'-OH normally present is missing. When these modified nucleotides are incorporated the addition of subsequent nucleotides is blocked, which leads to fluorescent DNA 'ladders' of differing lengths which can then be separated on polyacrylamide gels. Sequencing reactions were made as follows:

This method is commonly used for DNA sequencing based on chain termination by integration of dideoxynucleotides (ddNTPs) inhibiting the DNA polymerase. The reaction solution requires a DNA template, normal dNTPs, a DNA polymerase, a DNA primer and fluorescently labeled ddNTPs. At first, the DNA template is separated into two single strands by denaturation. During incubation of the single DNA strands and a specific primer with a DNA polymerase and ddNTPs, the ddNTPs act as chain-terminating inhibitors due to their lack of a 3´-hydroxyl group (3´-OH) that is needed for the phosphodiester bond between two nucleotides. Thus, whenever a ddNTP is incorporated into the growing oligonucleotide chain, the DNA polymerase cannot further extend the chain. This results in a mixture of DNA fragments of different length that all carry a terminal fluorescently labeled ddNTP. By capillary electrophoresis, the DNA fragments can be separated by size or length. The fluorescently labeled molecules are activated by a laser. The signals can be detected and recorded and correlate with the base sequence of the DNA strand.

For the present work, the sequencing reaction was performed by the BigDye v3 Terminator Kit (Thermo Scientific) and each reaction solution consisted of the following components:

1 µl of BigDye3.1

1 µl of 5x reaction buffer

1 µl of forward/reverse primer (1:10 dilution)

9 µl of purified PCR product

Samples were incubated for sequencing reaction under the following conditions

Table 11: Program for thermal cycler for sequencing reaction

Steps	Temperature [°C]	Time [s]	cycles
Initial denaturation	94	120	1
Denaturation	96	10	30
Amplification	60	120	30
Cooling	8	∞	1

:

4.6.4 Precipitation

Prior to capillary electrophoresis, DNA samples had to be removed from remaining proteins and salts by ethanol precipitation. For this purpose 2 µl of sodium acetate (NaAc), 8 µl of H_2O and 55 µl of 100% ethanol were added to each sample. The samples were then centrifuged at 14 000 rpm at 15°C for 30 minutes. Supernatant was discarded. Each 200 µl of 70% ethanol were added and samples were centrifuged again at 14 000 rpm at 15°C for 10 minutes. Supernatant was discarded. Then, samples were heated at 70°C for 5 to 10 minutes.

4.6.5 Capillary electrophoresis

Capillary electrophoresis was performed by a DNA sequencer (ABI Prism 3130 Genetic Analyzer), after ethanol precipitation. For this purpose, 15 µl of Hi-Di formamide were added to each sample. The sequences were compared with the reference sequences using the Sequencher program (Gene Codes).

5 Results

5.1.1 Mutational profile of the tumour

The unusual characteristic of this multifocal presentation, the evolutive highly aggressive and not classifiable tumour in this child, raised the question of a genetical underlying mechanism. An NGS approach was used to analyse in the primary tumour 94 genes and 284 SNPs that have been previously identified to be involved in cancer predisposition and/or oncogenesis. The Sequencing quality score (Q30) was 87.6%. The Mean Region Coverage Depth was of 618X. Three alterations had global allele frequency (from all populations of 1000 genomes data in April 2012) <=1% and were predicted to be deleterious by at least one algorithm (Table 1). These alterations were selected for validation by Sanger Sequencing in DNA extracted from the primary tumour and a brain metastasis and from blood all three genes are localised on chr.17.

Table 12: Genes identified by NGS

Gene	Variant	Chr	Transcript	Sift	PolyPhen	HGVSc	HGVSp	dbSNP ID	Allele Freq
TP53	A>A/G	17	NM_000546.5	deleterious(0)	probably_damaging(1)	NM_000546.5:c.737T>C	NP_000537.3:p.Met246Thr		0%
BRCA1	C>C/T	17	NM_007300.3	tolerated(0.24)	possibly_damaging(0.476)	NM_007300.3:c.3119G>A	NP_009231.2:p.Ser1040Asn	rs4986852	1%
RHBDF2	C>C/A	17	NM_024599.5	deleterious(0.03)	possibly_damaging(0.582)	NM_024599.5:c.1582G>T	NP_078875.4:p.Asp528Tyr	rs11553545	0,32 %

The Sift and the Polyphen algorithm were used to calculate the impact of the alterations on the protein function. The alterations were validated by Sanger sequencing on the tumour and the blood. The results of the validation (Yes/no) is indicated

The ***BRCA1*** **heterozygous missense mutation (c.3119G>A)** was validated in all three samples: from peripheral blood (PB), the primary abdominal tumour (no 109) and from the brain metastasis (no 217) at relapse (Figure 3A). Most significantly, while the mutation was heterozygote in the blood, the wild type allele was clearly decreased in the primary abdominal tumour and the metastasis. This alteration leads to the substitution of asparagine for serine at codon 1040 (p.Ser1040Asn) and has been previously described in breast cancer patients [38, 57-63]. In 1994, Friedman et al looked at 10 family pedigrees with breast cancer and ovarian cancer susceptibility, including 63 breast cancer patients, and described this variant as disease-causing mutation [64]. Further, the mutation is localised in the binding domain of *BRCA1* to *RAD51* (Figure 4A). The RAD51 protein is known to be crucial for the repair of DNA double-strand breaks in mammalian cells.[65]. In fact, the direct binding of *BRCA2* or *BRCA1* to the *RAD51* recombinase usually induces in the damaged cell homologous recombination repair.

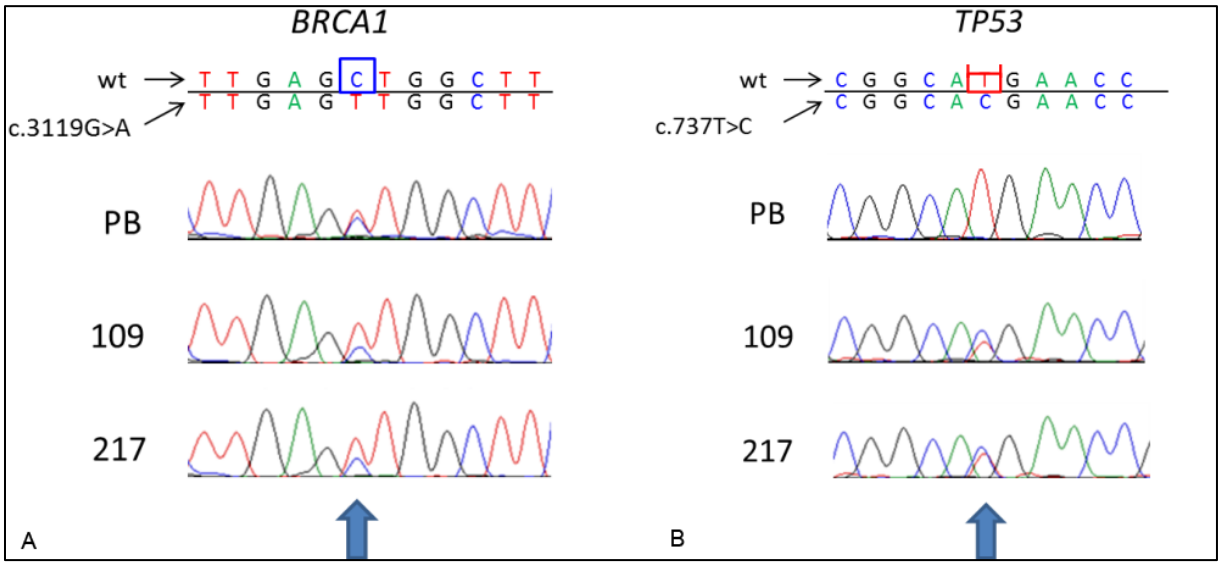
Screening the 5 homolog 2 (*RHBDF2)* gene, a **heterozygous germline mutation in rhomboid (c.1582G>T)** was detected**.** There is little published literature on mutations in *RHBDF2* (also known as RHBDL6 or iRhom2), which encodes for the inactive protease. RHBDF2 belongs to a family of seven transmembrane-spanning proteins called rhomboids, which were first identified in *Drosophila* and were shown to be serine intramembrane proteases linked with epidermal growth factor receptor (EGFR) signalling and mitochondrial remodelling [66]. Mutations have been described as underlying tylosis, a rare disorder in which affected individuals have a high risk of developing oesophageal squamous cell carcinoma [67].

The ***TP53*** **missense mutation (c737T>C)** (Figure 3B) has been previously described in one patient with colon cancer and a patient with breast cancer [68, 69]. This missense mutation leads to the substitution of threonine for methionine (p.Met246Thr) and is localised in the protein binding domain of *TP53* (Figure 4B).

The *BRCA1* c.3119G>A mutation has a minor allele frequency (MAF) of 1%, and is predicted to be damaging. The *RHBDF2* (c.1582G>T) has a MAF of 0.32% and is also predicted to be damaging. Allele variants are defined as rare when the minor allele frequency (MAF) is <1%, and as common variants those with MAF >5%. However, in the present patient, the wild type *BRCA1* allele was significantly reduced throughout the disease progression suggesting a non-casual correlation between the alteration

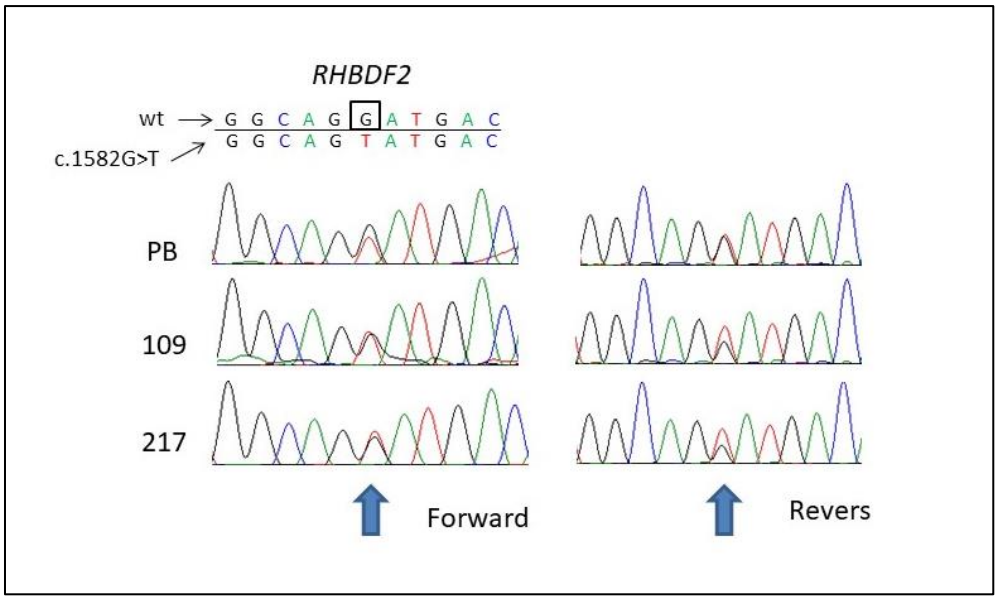
and the development of the disease despite the 1% allele frequency. Considering the allele frequency of the *BRCA1* germline mutation in our patient and the fact that it would be considered a polymorphism, **mutational signature analysis for BRCAness** was performed by the bioinformaticians of the University Tumor Center of Mainz (UCT) using Whole-Exome-Sequencing (WES) data available via INFORM and using the R-package "YASPA. Nine known mutational signatures with maximal exposure of Signature 12 (29.5%), Signature 8 (22.5%) and Signature 3 (21.9%) were detected. Signature 3 and signature 8 have been linked to *BRCA2* and *PALB2* germline mutations and generally with homologous-recombination deficiency [70]. Signature 12 has been found in liver cancer but its role in tumourigenesis remains unknown [71]. Signature 3 associates strongly with elevated numbers of large insertions and deletions. Further, copy number analysis of signature 3 revealed a highly fragmented DNA profile with loss of larger chromosome fragments but without loss of Chr17. Notably, specific signatures like signature 3 and 8 that are linked to homologous-recombination deficiency, have been shown to occur in many different cancer types and not be specific to Hereditary Breast and Ovarian Cancers HBOC [72]. In the absence of either BRCA1 or BRCA2, a characteristic mutational signature consisting of elevated DNA deletions and short, tandem DNA repeats at the break points of the deletion has been described by Tutt et all [73] and by Xia et al [74]. The present results suggest that the identification of heterozygous germline mutations in tumour suppressor genes like *BRCA1* despite the allele frequency, should lead to further analysis concerning *BRCAness*.

Figure 5: Validation of the *BRCA1* and *TP53* alterations



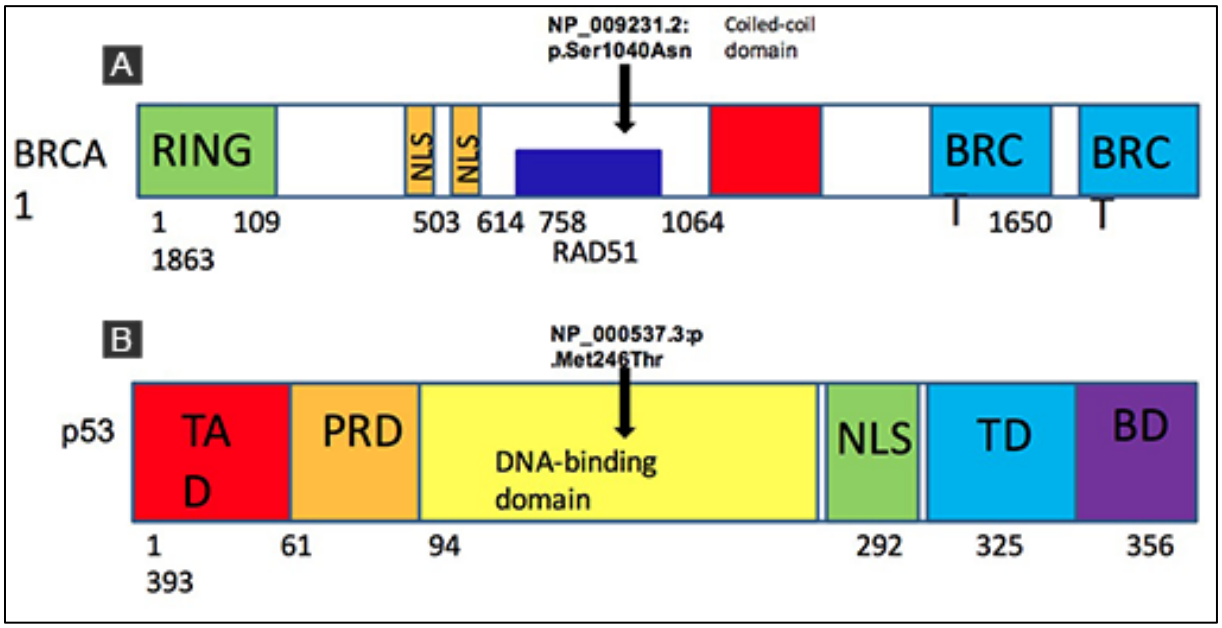
BRCA1 [A] and TP53 [B] were analysed by Sanger Sequencing in the genomic DNA extracted from Peripheral Blood (PB), the primary tumour at the time of the first surgery (no 109) and from a brain metastasis (no 217). Own figure.

Figure 6: Validation of the RHBDF2 mutation



RHBDF2 was analysed by Sanger Sequencing in the genomic DNA extracted from Peripheral Blood (PB), the primary tumour at the time of the first surgery (no 109) and from a brain metastasis (no 217). Own figure.

Figure 7: Localisation of BRCA1: p.Ser1040Asn and TP53 p.Met246Thr



With respect to the BRCA1 and TP53 domains [A] : BRCA1 contains a RING domain, two nuclear localisation sequences (NLS), binding sites for several proteins like RAD51, a coiled-coil domain and two BRCA1 C Terminus domain (BRCT). The possibly damaging BRCA1 missense mutation described here is localised in the RAD51 binding site. Modified after Clark, 2012 [75]. [B] TP53 contains a transcriptional activation domain (TAD), a prolin rich domain (PRD), the DNA-binding domain in which the missense mutation is localised, a tetramerisation (TD) and a basic domain (BD). Modified after Qian, 2013 [76].

6 Discussion

The unusual characteristic of this multifocal presentation, the evolutive highly aggressive and non-classifiable tumour in this young child, raised the question of an unknown genetic underlying mechanism. To address this question, Next-Generation Sequencing was employed to evaluate the mutational profile from the primary lesion and later of a metastatic lesion.

Several previous studies in different ethnic groups described and evaluated the risk association between cancer predisposition and the missense mutation c.3119G>A in *BRCA1* with conflicting results. The first description and association of the mutation with cancer predisposition was described by Friedman et al. In their study, the mutation penetrance was high in an affected family (six family members with early onset breast cancer, two of them had bilateral disease) and the mutation was not found in 120 control chromosomes [64]**.** According to the algorithms used in this study, the germline mutation in the *BRCA1* is possibly damaging. However, several studies describe this specific mutation as a polymorphism as it occurs in different members of a population [58, 60, 64]**.** The mutation is localised in the largest *BRCA1* exon which contains protein binding domains for a number of diverse proteins and which is frequently mutated in cancer patients [75]**.** Interestingly, the mutation is localised in the *RAD51* binding site of *BRCA1*. *RAD51* is considered a key factor of homologous recombination (HRR) and double-stranded-break repair [77]**.** It is well known that the interaction between *RAD51* proteins and *BRCA1* is essential for the function of *BRCA1* as tumour suppressor and therefore an effective DNA repair [78, 79]**.** It has been shown that when HRR is defective or absent because of BRCA1/2 mutations, BRCAness phenotype, other forms of DNA repair such as non-homologous end joining (NHEJ) are used to repair double-strand-breaks [65] which may result in the introduction of DNA mutations, particularly deletions [80]. The germline allele frequency of the missense mutation c.3119G>A in *BRCA1* is around 1%. Therefore, considering only pathogenicity criteria used in the literature, this mutation would be considered non-pathogenic because it is “too common” in the general population. For decades, the use of the 1% frequency threshold to develop population models has been used. However, with the advent of new sequencing technologies and the availability of large data on individuals, a very different picture of population dynamics has begun to emerge. Mutations that were

considered to be rare in a population have been found to exceed the frequency threshold of 1%. Moreover, a disease-causing mutation in one population may be harmless in another, and vice versa [81]. Thus, the 1% cut-off may be not an effective parameter to identify cancer predisposition loci and further criteria should be introduced.

Following the dynamic of the mutated germline allele in the tumour, possibly in combination with somatic mutations, may suggest new criteria to identify relevant cancer predisposition loci. First, in the present work, it was observed that the *BRCA1* mutated allele was maintained while the wild type allele was reduced in the primary tumour and in the brain metastasis suggesting that tumour cells without the normal allele had a growth advantage. Further, indicating that one defective germline copy of *BRCA1* is enough to cause a cancer predisposition, the second allele being lost in the tumour cells of the affected individuals, as generally accepted for autosomal dominant cancer predisposition syndromes.

Second, in the present analysis a non-fortuitous association between the germline *BRCA1* mutation and the alteration of *TP53* was suspected. It is recognised that within a population, a germline mutation (first hit) may predispose a subset of patients to a second, somatic mutation, whose effects will create the diseased phenotype. Multiple studies suggest that the loss of *BRCA1* cooperates with the loss of *TP53* function in carcinogenesis [56, 82]**.** Decreased TP53 leads to a reduced apoptosis and allows BRCA1 defective cells to proliferate [19]. Accompanying somatic alterations of *TP53* in *BRCA1* carrier, have been already described several times in breast and ovarian cancer patients, and are known to be responsible for a more aggressive tumour progression due to a transcriptional regulation of *BRCA1* [19, 20]. Whether the extremely aggressive, multifocal and metastatic presentation of the tumour in the present patient was due to the synergistic effect of the germline mutation in *BRCA1* and the altered *TP53* is speculative.

Considering the allele frequency of the *BRCA1* germline mutation in the present patient and the fact that it would be considered a polymorphism, the mutational signature of the tumour for BRCAness was analysed. The term of BRCAness or “signature 3” is used to describe a phenotype in which some sporadic tumours show molecular abnormalities similar to BRCA-mutated tumours. In the last years, various mechanisms

that can lead to the BRCAness phenotype have been described. Signature 3 was found in patients carrying germline and somatic BRCA1/2 mutations as in sporadic tumours of patients with non-mutated *BRCA1/2* genes in which the epigenetic silencing of a BRCA gene was caused by promoter methylation and gene deletion.

In the present case, the coexistence of three mutational forces (mechanism); the *BRCA1* biallelic inactivation, the presence of two mutational signatures (8 and 3), together with the presence of large structural variants, suggest that the c.3119G>A *BRCA1* mutation may have played a role in the development of this aggressive tumour.

Additionally, a germline mutation in *RHBDF2* was detected which has been described to be causative for tylosis, a rare disorder characterized by risk of developing oesophageal squamous cell carcinoma [67]. It is known that *RHBDF2* plays a key role in the secretion of tumour necrosis factor alpha [83] and is involved in the epidermal growth factor receptor (EGFR) signaling pathway [84], but its role in cancer development has not yet been fully elucidated. According to the Pecan portal , *RHBDH* mutations have been detected in different cancer subtypes. According to cBioPortal [85], somatic mutations are found in 0,5 % of 42,049 samples in 158 studies with no hot spots regions. Interestingly, the three mutations detected in the present childhood cancer patient, were all localised on chromosome 17. The loss of chromosome 17 and its tumour suppressor genes (TSG) has been previously described as a common occurrence in ovarian cancer, as suggested by loss of heterozygosity (LOH) studies. Moreover, the *17q21.31* region harbours the TSG *BRCA1.* Further, Wojnarowicz et al. [86] proposed *RHBDF2* to be a new TSG localised in region *17q25,* close to *17q21.31,* to play a key role in the development of ovarian cancer.

The finding of these two germline mutations localised on the long arm of chromosome 17 raises the question as to whether they have had synergistic effects on a selective advantage in the development of this aggressive metastatic cancer.

Despite multimodal therapy, including tumour resection, multi-agent chemotherapy and radiotherapy, the child developed transperitoneal spread and brain metastasis. The identification of *BRCA1* as cancer predisposition gene in paediatric patients opens the way to potential new treatment options. Many therapy-optimising studies in *BRCA1/2*-driven cancers in the adult population are being performed using

combination therapy with platinum-based chemotherapy and poly(ADP-ribose) polymerase (PARP) inhibitors [87, 88]**.** Previous studies have shown that BRCA-mutated cancer cells are characterised by a deficient DNA repair mechanism by homologous recombination (HR), consequently they are vulnerable to DNA-damaging cytotoxic agents, such as platinating drugs especially when combined to PARP inhibitors [89]**.** Although stratified, targeted treatment is currently rarely incorporated into first line therapy, it is more and more applied in case of standard treatment failure especially when the tumour harbours known potential genetic targets. The combination of platinating drugs with PARP-inhibitor therapy was considered in the present childhood cancer patient when the infant developed brain metastasis because beside the good explored cytotoxicity, it is known to have a good capacity to penetrate blood-brain barrier [90, 91]**.** Unfortunately, the patient died of tumour progression, thus a targeted therapy with PARP inhibitors was not attempted.

The present findings revealed the unexpected presence of a potential disease-causing mutation in an adult-onset-cancer-predisposing gene and address the important and challenging topic of it's probably underestimated presence in the paediatric patient population. As in many paediatric cancer predisposition syndromes, in the case of the described child, there were no morphological anomalies or cancer specific genetic alterations that could have helped in the initial diagnostic. Furthermore, the family history was not indicative for a cancer predisposition syndrome. This pointing out, how in children the suspicion of an underlying genetic disorder often comes along with the diagnosis of a rare cancer entity and not vice versa. The knowledge of the family history is usually a very helpful tool for the detection of adult-onset-cancer-predisposition syndromes as *BRCA1* and *BRCA2*, but it is rarely informative when the underlying germline mutation causes a non-common cancer type at an "unusual" young age. This confirms how the clinical presentation of germline mutations is variable and the diagnosis may not be obvious immediately even when the affected gene is a known cancer predisposing gene. Pointing out the need of a more systematic genetic counselling, particularly for patients with uncommon presentation and high-risk entities.

The evidence gathered in this study appears to support the hypothesis that the percentage of childhood cancers caused by underlying genetic mutations is probably underestimated, as mutational analysis of these genes is not usually performed in this population. The identification of a tumour predisposition syndrome in paediatric cancer

patients is paramount for optimal care because it can affect management of cancer treatment and aftercare. Thus, despite the previous discussed ethical considerations, introducing *BRCA* mutational screening in paediatric patients with non-classifiable tumours, advanced disease and poor therapy response to standard treatment, could have therapeutic and clinical implications in the future.

The work of this thesis points out the crucial role for a more intense analysis of the individual's tumour tissue and comparison with the patient's DNA on a molecular level. Comparing the genome of the tumour with the germline genome of the paediatric cancer patient more somatic alterations in the tumour might be identified and driving mutations identified. Now that the new sequencing technologies have dramatically reduced the cost of sequencing, the comparison of the tumour DNA with the germline DNA of the same patient may allow a personalised identification of a cancer-predisposition-syndrome beside of the 1% cut-off. The characterisation of clinical features, along with the molecular characteristics of the patient's tumour, could allow a more specific genetic counselling and testing in the future. This could lead to improved surveillance guidance which could be implemented in subsequent standard-of-care trials.

This also highlights the need for more personalised profiling in the paediatric cancer population, both to increase the identification of at-risk patients needing a close follow up, and who might benefit from less harmful targeted therapies. Indeed, most malignancies associated with a cancer predisposition syndrome require specific treatment strategies and screening for associated subsequent malignancies.

Finally, for the detection of further susceptibility loci, genome-wide studies using lager numbers of cases and controls along with the combination of results across multiple studies for a better understanding of tumour biology and therapy-resistance mechanism are needed. Although the contribution of low penetrance and high-frequency alleles remains unknown, high-throughput sequencing based methods are entering more and more clinical use and there is hope that an era of actual personalised medicine will become reality in the near future.

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8 Acknowledgement

9 Curriculum vitae