Shared Copy Number Variation in Simultaneous Nephroblastoma and Neuroblastoma due to Fanconi Anemia

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Key Words

Copy number variation · Fanconi anemia · Nephroblastoma · Neuroblastoma · *PALB2* mutation

Abstract

Concurrent emergence of nephroblastoma (Wilms Tumor; WT) and neuroblastoma (NB) is rare and mostly observed in patients with severe subtypes of Fanconi anemia (FA) with or without VACTER-L association (VL). We investigated the hypothesis that early consequences of genomic instability result in shared regions with copy number variation in different precursor cells that originate distinct embryonal tumors. We observed a newborn girl with FA and VL (aplasia of the thumbs, cloacal atresia (urogenital sinus), tethered cord at L3/L4, muscular ventricular septum defect, and horseshoe-kidney with a single ureter) who simultaneously acquired an epithelial-type WT in the left portion of the kidney and a poorly differentiated adrenal NB in infancy. A novel homozygous germline frameshift mutation in *PALB2* (c.1676_c1677delAAinsG) leading to protein truncation

(pGln526ArgfsX1) inherited from consanguineous parents formed the genetic basis of FA-N. Spontaneous and induced chromosomal instability was detected in the majority of cells analyzed from peripheral lymphocytes, bone marrow, and cultured fibroblasts. Bone marrow cells also showed complex chromosome rearrangements consistent with the myelodysplastic syndrome at 11 months of age. Array-comparative genomic hybridization analyses of both WT and NB showed shared gains or amplifications within the chromosomal regions 11p15.5 and 17q21.31-q25.3, including genes that are reportedly implicated in tumor development such as *IGF2*, *H19*, *WT2*, *BIRC5*, and *HRAS*.

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Nephroblastoma (Wilms Tumor; WT) and neuroblastoma (NB) are the most common solid tumors in children. However, the simultaneous occurrence of these and similar tumors is rare and observed mostly in patients with severe subtypes D1 and N of Fanconi anemia (FA; MIM: 227650). While the high incidence of such tumors

in FA-D1 and -N patients with or without VACTER-L association (VL; MIM: 192350) reflects their severe genomic instability, the genetic mechanisms leading to concurrent tumor development are largely unclear. It has been established that heterogeneity of the manifestations shown by FA patients stems from rather varied phenotype-genotype correlations, yet tumor development within specific FA subtypes is a certainty [Nevelling et al., 2009]. Accordingly, biallelic mutations in *BRCA2/FANCD1* or *PALB2/FANCN* predispose to 'embryonal' or 'developmental' types of tumors in the first years of life, particularly WT, NB, hepatoblastoma, and medulloblastoma [Abbondanzo et al., 1986; Berrebi et al., 2006; Kopie et al., 2011].

However, a former hypothesis that mutations in FA genes would be found in FA-typical neoplasms in the general population has not been confirmed, suggesting that not FA gene mutations alone but additional, more or less randomly occurring events such as amplification or deletion of oncogenes or tumor suppressor genes, respectively, must be involved in tumor development in FA patients of subtypes D1 or N. An example is the early report on the co-occurrence of WT and NB in an infant with FA with gain of chromosome 17q material in the NB which is considered an important prognosis factor [Bissig et al., 2002].

The aim of the present study was the analysis of somatic genomic alterations in simultaneously arisen WT and NB of a girl with FA-N, incomplete VL, and extreme chromosomal instability. This investigation addresses the question if and which genomic abnormalities besides the constitutional FA gene mutations might be present in both tumors, aiming to identify possible shared aberrations in key candidate genes for tumor development.

Clinical Summary

The female patient was referred to the pediatric surgery department at Ulm University immediately after birth due to a complex association of malformations. The child showed an incomplete VL with sacral dysplasia and tethered cord at L3/L4, ventricular septum defect, supralevator anal atresia, uterine bicorn dysplasia, vaginal atresia, fused and severely malformed thumbs, and a pelvic horseshoe kidney encroaching the aorta and inferior cava vein. Shortly after birth the patient developed glomerular proteinuria, elevated blood pressure, and failure to thrive. In the familial history, there was a clustering of tumors (mostly carcinomas of the lung and breast) on the maternal side of the family.

After initial stabilization over 3 months, posterior-sagittal anorectoplasty was performed, and the girl was subsequently followed-up in the pediatric outpatient clinic. At the age of 10 months (during a routine ecography) a mass measuring $4.0 \times 5.0 \times 5.6$

cm in the horseshoe kidney was detected. Subsequent imaging studies (MRI) demonstrated a tumor originating from the left portion of the kidney with marked central necrosis, compatible with a WT compressing the adjacent structures. MRI scans of the brain and thorax did not show any other primary tumors or metastases at that time. Diagnostic differential considerations precluded a NB or a germ cell tumor due to normal levels of β -HCG, AFP, and catecholamines.

Based on the diagnosis of WT, the patient was enrolled for the SIOP-2001/GPOH protocol [2001] for neo-adjuvant therapy and received pre-operatively 4 cycles of chemotherapy (Actinomycin-D and Vincristine) which led to a significant reduction of the tumor size. The initial chemotherapy was followed by the surgical resection of the neoplasm at the age of 11 months. Surgery consisted of nephrectomy with complete resection of the left kidney portion and staging lymph node resection of the left perirenal region, including the left adrenal gland containing a second tumor mass that must have emerged rapidly pre-operatively. The patient recovered without complications and received postoperative chemotherapy according to the above protocol. Subsequently, she developed a myelodysplastic syndrome which required additional therapy and was discharged from the hospital not until 8 months after tumor resection. Informed written consent of the parents including permission for further genetic studies of the tumors was obtained at this time, in accordance with the guidelines of the Ethical Review Committee of the University of Ulm.

One year after the primary tumor resection the patient was acutely admitted to the intensive care unit with clinical symptoms of increased intracranial pressure. MRI showed a large tumor mass ($7.5 \times 6.4 \times 8.7$ cm) within the cerebellum with severe compression of the brainstem. Although adequate treatment was initiated, the patient died within a few hours. Pathological diagnosis of the tumor was not feasible since the parents did not authorize an autopsy, yet the tumor presentation on MRI images was suggestive of medulloblastoma which also seemed the most likely tumor type given the localization, the relation to the previously developed tumors, and the spectrum of infantile cancers reported in FA subtype D1 and N patients. Formally, a primary germ-cell tumor, a meningeal sarcoma, or a metastatic NB were considered but appeared extremely unlikely.

Materials and Methods

Histopathology

For histopathological examination, formalin-fixed and paraffin-embedded sections of the tumors were stained with hematoxylin and eosin according to standard protocols.

Immunohistochemistry

Paraffin sections of about $2 \mu\text{m}$ were obtained and stained according to standard protocols. Briefly, polyclonal FLI-1 serum (RB9295; Medac, Wesel, Germany) was diluted 1:100 in phosphate saline buffer, and aliquots of $100 \mu\text{l}$ were incubated on the deparaffined slides. The slides were pre-treated for antigen retrieval (microwave oven for 20 min in citrate buffer 20 mM, pH 6). For antigen detection system we used the Dako detection kit K5005 (Dako, Glostrup, Denmark); as positive control, paraffin sections of a Ewing's sarcoma were used.

Karyotyping of Peripheral Blood Cells

Cytogenetic studies were performed on phytohaemagglutinin (PHA)-stimulated lymphocytes from peripheral blood obtained at the age of 9 days and 7.5 months, respectively, cultured for 72 h, and prepared by standard procedures. Cytogenetic analysis on fibroblasts was performed on cultured fibroblasts from a skin biopsy taken at the age of 8.5 months. Metaphase chromosomes were prepared by standard techniques.

Mitomycin C-Induced Chromosome Breakage Analysis

Metaphases were prepared from whole blood cell cultures. Aliquots of 0.8 ml heparinized blood and 0.2 ml PHA were added to each 10 ml RPMI 1640 medium containing GlutaMAX and 15% FBS (GIBCO Invitrogen, Darmstadt, Germany). Duplicate cultures with final concentrations of 0, 50, and 100 ng/ml mitomycin C (Medac) were set up no later than 2 days after blood samples were drawn and maintained for 72 h incubation at 37°C. For the last 45 min, 80 µl Colcemid solution (10 µg/ml; Invitrogen) were added. Harvest was followed by hypotonic treatment of the cells with 0.075 M KCl and fixation with methanol:acetic acid 3:1. Slides were stained in 5% aqueous Giemsa and screened on an Axioskop Imager A1 (Carl Zeiss, Jena, Germany). Chromatid breaks were rated as 1, radial figures as 2 or more break events. Chromosome-type damage was counted as 1, 2, or more breaks depending on the mode of origin of a specific aberration. Cells were classified into a scale from 0 to ≥10 breaks/metaphase resulting in histograms of break distributions. Chromosome breakage rates were calculated as breaks/metaphase on the basis of at least 50 analyzed mitoses.

Karyotyping of Bone Marrow Aspirate

After short-term culture for 20 h, metaphases from bone marrow aspirate were prepared according to standard procedures. Fluorescence R-banding using chromomycin A3 and methyl-green was performed as reported in detail earlier [Perel et al., 1998]. Karyotypes were described in compliance with the International System for Human Cytogenetic Nomenclature [ISCN, 2009]. A complex karyotype was defined as 3 or more clonal aberrations including at least one structural aberration [Schlegelberger et al., 1999; Göhring et al., 2010].

PALB2/FANCN Sequencing

High molecular DNA was extracted from blood cells or fibroblasts using a modified salting-out method [Miller et al., 1988]. We used primers and PCR conditions previously reported by Reid et al. [2007] for amplification of the *FANCN* exons and adjacent intronic regions from genomic DNA. For mutation analysis, we designed a pair of semi-nested primers, 4F: 5'-CACAGGACAA-CCAAGTTCAA-3' (Tm 58°C) and 4R: 5'-AAGGAAGTGCC-AGGCAAATA-3' (Tm 58°C). For sequencing of PCR products, we used the same primers as for amplification, the BigDye Terminator v1.1 cycle sequencing kit, and a 3730 Genetic Analyzer (Applied Biosystems, Darmstadt, Germany).

WT1 Mutation Analyses

All *WT1* exons, except exons 8 and 9 were analyzed for mutations with denaturing high performance liquid chromatography (DHPLC) (WAVE, Transgenomic, Glasgow, UK) as described previously [Royer-Pokora et al., 2010]. Exons 8 and 9 were sequenced directly. Exons demonstrating an aberrant pattern on

DHPLC were sequenced on an ABI 3100 automated capillary DNA sequencer (Applied Biosystems) using cycle sequencing procedure with the BigDye terminator kit.

Fluorescence in situ Hybridization (FISH) for MYCN Amplification and 1p Deletion Studies (NB)

FISH technique was applied using a DNA probe Vysis CEP 2 SO as a reference and the probe LSI N-MYC SG hybridizing on chromosome 2p24 (Abbott Molecular Inc., Des Plaines, Ill., USA) in order to count the number of *MYCN* copies in relation to the number of chromosomes 2. FISH was performed as a dual color procedure following the manufacturer's instructions. According to the recommendations of the European Neuroblastoma Quality Assessment group (ENQUA) [Ambros and Ambros, 2001], amplification was defined as more than the 4-fold increase of *MYCN* signals in respect to the number of chromosomes 2. Additional copies exceeding up to 4-fold the number of reference signals on the chromosomal arm were defined as *MYCN*-gain. The chromosome 1p microdeletion region was assayed with the dual-color probe Vysis LSI 1p36/LSI 1q25 (Abbott Molecular). According to ENQUA guidelines, 1p36 aberrations were defined as (a) deletion: monosomy of the specific region, ratio of the reference 1q region to the specific regions 2:1, 3:1, and so forth; (b) imbalance: at least 2 intact copies of the chromosome and additional copies with deletions in the specific region (i.e. no monosomy), ratio of reference 1q region to specific regions 3:2, 4:2, 4:3, and so forth. The presence of an EWSR1/FL11 fusion protein was probed with the Vysis EWSR1 Dual Color Break Apart FISH probe kit (Abbott Molecular). At least 100 nuclei were counted for each analysis. Conventional fluorescence images were taken with a Leica DMRA microscope equipped with a JVC KY F-75 digital camera and DISKUS software (Hilgers, Königswinter, Germany).

Array Comparative Genomic Hybridization (aCGH)

Our assay utilized comparative hybridization of labeled probe DNA (patient) in competition with a genomic reference DNA (human male genomic DNA, Promega, Madison, Wisc., USA) on an array containing 170,334 specific oligonucleotides (Agilent Human Genome CGH microarray 180K, Agilent Inc., Santa Clara, Calif., USA) with an average genomic spacing of 17,665 kb between the probes. Aberrations were classified as 'loss' when there was half signal intensity for at least 5 contiguous oligonucleotides (88.3 kb in the mean), allowing statistically for the detection of deletions <100 kb, or as 'gain' when there was double signal intensity for 10 or more consecutive probes (176.6 kb in the mean). Such abnormalities of copy number variation (CNV) were classified as 'true' when the suspected regions were annotated in the Database of Genomic Variants (<http://projects.tcag.ca/variation>). Our results were interpreted on the basis of the oligonucleotide positions provided by Agilent referring to the UCSC Genome Browser on Human Mar. 2006 (NCBI36/hg 18).

Results

Histopathological examination of the kidney tumor showed an epithelial-rich type WT (intermediate risk group) with regressive changes after chemotherapy. In

the left adrenal gland a poorly differentiated, stroma-poor NB was detected (fig. 1).

Conventional chromosomal analysis of peripheral blood lymphocytes revealed a 46,XX female karyotype with spontaneous chromosome instability in the majority of metaphases. In lymphocyte cultures from the first analyzed blood sample of the patient only 3 metaphases had a normal karyotype, whereas 36 metaphases showed a variable pattern of chromatid- and isochromatid-type aberrations with a total of 23 chromatid gaps, 50 chromatid breaks, 1 chromosome gap, 7 chromosome breaks, and 50 structural aberrant chromosomes including an identifiable translocation, a deletion, a strongly modified derivative chromosome, and an unidentifiable marker chromosome. This observation further suggested a chromosome instability disorder. In cultures from the second blood sample only 22 metaphases had a normal karyotype, whereas 71 metaphases showed a total of 42 chromatid gaps, 77 chromatid breaks, 6 triradial figures, 5 chromosome gaps, 2 chromosome breaks, and 66 structural aberrant chromosomes. In chromosome preparations from the fibroblast cultures only 31 metaphases had a normal karyotype, while 94 metaphases showed a total of 19 chromatid gaps, 28 chromatid breaks, 2 triradial figures, 1 chromosome break, and 65 structural aberrant chromosomes. These observations were considered consistent with FA.

Quantitative analysis of chromosome instability in PHA-stimulated cultures of peripheral lymphocytes revealed a high spontaneous breakage index of 0.33 breaks per metaphase (normal rate ≤ 0.02) and excessively increased chromosome breakage after induction of cultured cells with mitomycin C, in which there were >10 breaks in 100% of the metaphases following exposure to 50 ng/ml (normal rate ≤ 0.03 per metaphase) and 100 ng/ml (normal rate ≤ 0.06 breaks per metaphase) (fig. 2).

At the time of diagnosis of myelodysplastic syndrome, bone marrow analysis revealed structurally complex aberrations with the karyotype designation: 46,XX,add(1)(p33),add(2)(q37),dup(3)(q28q24),del(5)(q14q34),-7,add(9)(p21),del(20)(p12),+mar[cp13]/46,XX[2].nuc ish 3q26(EVI1 $\times 3$)[51/100],cen7(CEP7 $\times 2$),7q31(D7S486 $\times 1$)[79/100],cen8(CEP8 $\times 2$)[100]. Other abnormalities such as chromatid and chromosome gaps and breaks and triradial figures were observed.

The unusually high breakage rate suggested that the patient belonged to either the FA subtype D1 or N. Mutation analysis carried out in DNA from peripheral blood using exon-scanning-sequencing of the complete coding

Fig. 1. Histology of the analyzed nephroblastoma WT (A) and NB (B); hematoxylin-eosin staining. The WT is of the epithelial type with a characteristic tubular growth pattern. Nuclei (insert) have condensed chromatin. The NB is poorly differentiated with few intermingled collagen bundles. Nuclei (insert) have some small nucleoli and show slight differences in diameters.

region of the *BRCA2/FANCD1* gene (Entrez Gene: 675; OMIM: 600185) showed no pathologic sequence variants. However, sequencing of the 13 exons of the *PALB2/FANCN* gene (Entrez Gene: 79728; OMIM: 610335) revealed a novel homozygous frameshift mutation c.1676_1677delinsG (c.1676_1677delAAinsG) which leads to a premature stop codon and protein truncation (p.Gln526ArgfsX1). Father, mother, and brother of the patient were heterozygous for this mutation (fig. 3).

Fig. 2. Induction with mitomycin C (MMC) provokes extremely high chromosome breakage rates in lymphocyte cultures from the present patient. Note the increased breakage even without mitomycin C induction.

In addition, the patient was heterozygous for the common polymorphism in the 5'-UTR of *WT1*, c.1-7G>T (rs2234583). The NB revealed 2–10 signals pro nucleus for both the control region and *MYCN*, reflecting aneuploidy. Some cell nuclei showed a small excess (up to 4-fold) of *MYCN* signals over control signals, suggesting the possibility that subclones with *MYCN* amplification had emerged during tumor progression. Screening for chromosome 1p deletions in the NB was only possible in few cell nuclei ($n = 61$). The chromosome 1p36/q25 regions were found mostly disomic (data not shown). Immunohistochemical staining failed to detect an EWSR1/FL11 fusion protein in either neoplasm, indicating that the EWSR1/FL11 rearrangement was not involved in tumor development.

aCGH revealed a complex pattern of amplifications or gains and deletions. These results were obtained by comparing tumor with normal control DNA and generally were highly significant ($p < 0.05$; table 1, fig. 4).

The information on gene location within the regions of CNV provided with the array was confirmed by com-

parison with the Ensembl (http://www.ensembl.org/Homo_sapiens/Info/Index) and NCBI (<http://ncbi.nlm.nih.gov>) databases. Those genes were cross-referenced to the literature on genes potentially associated with the development and/or progression of NB (455 genes thus far described in the literature) or WT (82 genes). The genes most important for these processes in terms of our study were considered those whose CNV was shared by both tumors.

Amplification 11p15.5 (NB, WT)

This amplification encompasses the most common cluster for loss-of-heterozygosity (LOH) in WT between markers D11S1318–D11S1288 and D11S1338–D11S1323 [Karnik et al., 1998] and comprises several WT and NB key genes, for example *IGF2* (Entrez Gene: 3481; OMIM: 147470). In addition, epigenetic alterations in the 11p15.5 gene cluster are frequently associated with human cancers including WT [Astuti et al., 2005, Barth et al., 2000]. *H19* (Entrez Gene: 283120; OMIM: 103280), a gene in that region, expresses a non-coding RNA and functions as a

Fig. 3. *PALB2/FANCN* sequencing analysis and pedigree. **A** Direct sequencing of exon 4 PCR products from genomic DNA of patient blood revealed the homozygous frameshift mutation c.1676_1677delAAinsG predicted to lead to protein truncation (p.Gln526ArgfsX). The arrow points to the insertion of G while the circle encompasses the AA dinucleotide in a normal control *PALB2* sequence which is deleted in the patient. **B** Pedigree of the family. Both parents and the brother of the index patient are heterozygous for the c.1676_1677delAAinsG mutation, suggesting consanguinity of the parents.

tumor suppressor. Variants in *H19* along with overexpression of *IGF2* have been reported to be a characteristic feature of various embryonal tumors, including WT. This is possibly due to alterations of the DNA modification machinery which leads to the activation of imprinted genes in the emergence of blastomas [Hubertus et al., 2011]. *HRAS* (Entrez Gene: 3265; OMIM: 190020) belongs to the family of *RAS* oncogenes and encodes a protein with intrinsic GTPase activity, functional in several signal transduction pathways. Variants of this gene and/or decreased expression of *HRAS* have been associated with increased tumor rates, including NB with poor prognosis [Kusafuka et al., 1995]. *IGF2AS* (Entrez Gene: 51214; OMIM: 610146) is paternally imprinted and encodes an antisense transcript of the *IGF2* gene which is overexpressed in WT [Vu et al., 2003]. *BRSK2* (Entrez Gene: 9024; OMIM: 609236) is a gene whose expression has been shown to be upregulated in several tumors such as pancreatic ductal carcinoma and possibly NB [Niu et al., 2010]. *CTSD* (Entrez Gene: 1509; OMIM: 116840) is

associated with the *MYCN*-dependent regulation of drug resistance of NB and the development of other tumors such as breast cancer [Sagulenko et al., 2008].

Amplification 17q21.31-q25.3 (NB, WT)

This amplification is seen in ca. 47% of stage IV NB patients and is associated with poor prognosis [Lavarino et al., 2009]. LOH of this cluster includes several key genes like *GALR2* (Entrez Gene: 8811; OMIM: 603691) which encodes a galanin receptor involved in signaling through the phospholipase C/protein kinase C pathway; its expression has been shown to be decreased in NB [Berger et al., 2002]. *BIRC5* (Entrez Gene: 332; OMIM: 603352) inhibits apoptosis and has extensively been analysed in NB, since its overexpression is stage-dependent, associated with poor prognosis and with the expression of the transcription factor *E2F1*, suggesting that *BIRC5* is induced via functional cooperation between *MYCN* and *E2F1* [Eckerle et al., 2009]. *NME1* (Entrez Gene: 4830; OMIM: 156490) mutations have been identified in aggressive NB

Table 1. Quantification and significance of CNV in the neuroblastoma and the nephroblastoma in the patient

Chromosome	NB				WT			
	cytoband	chromatid gain	deletion	p value ^a	cytoband	chromatid gain/ amplification	deletion	p value ^a
1	NA				p36.33-p12	–	–0.807153	0.000
2	p25.2-p21	+0.509640	–	0.000	NA			
3	NA				q26.2-q29	+0.628710	–	0.000
4	p16.3-p15.31	–	–0.710137	0.000	NA			
5	NA				p15.33-p14.2	–	–0.865332	0.000
6	p25.3-p21.33	–	–0.710137	1.88 ^{E-300}	NA			
7	q11.23-q36.3	+0.501730	–	0.000	NA			
8	NA				p23.3-p21.2	–	–0.905363	2.02 ^{E-15}
9	p24.3-p11.2	–	–0.321282	1.50 ^{E-172}	NA			
10	q22.3-q24.2	–	–0.672336	0.000	NA			
11	<u>p15.5</u>	<u>+0.342452</u>	–	2.83 ^{E-11}	<u>p15.5-p15.4</u>	<u>0.673658</u>	–	2.96 ^{E-75}
	q24.3-q25	+0.476737	–	6.14 ^{E-60}	p14.3-q11	–	–0.836182	0.000
					q11-q12.2	–	–0.437962	9.10 ^{E-50}
					q13.1-q25	–	–0.786518	0.000
12	NA				NA			
13	NA				NA			
14	q22.1-q32.33	–	–0.668273	0.000	q24.1-q32.33	–	–0.768244	0.000
					q32.33	+1.235929	–	2.51 ^{E-22}
15	q11.2	–	–0.784262	5.93 ^{E-11}	q11.2	–	–0.669122	5.86 ^{E-16}
16	p13.3-p13.13	–	–0.656460	6.84 ^{E-232}	p13.3	+0.920840	–	0.000
	NA				p13.3	+3.086544	–	1.89 ^{E-25}
17	NA				p13.3-p12	–	–0.660091	0.000
	<u>q21.2-q25.3</u>	<u>+0.573892</u>	–	0.000	<u>q21.31-q25.3</u>	<u>+1.133355</u>	–	0.000
	NA				q25.3	+2.865505	–	4.18 ^{E-11}
	NA							
18	NA				NA			
19	p13.3-p13.11	–	–0.630920	0.000	p13.3	–	–0.456250	2.49 ^{E-25}
	p13.11	+1.507263	–	2.05 ^{E-07}	q13.33	+0.935413	–	5.54 ^{E-11}
	q13.42-q13.43	–	–0.626082	9.44 ^{E-92}	q13.32-q13.43	–	–0.621049	0.000
20	NA				p13.3-p11.23	–	–0.586223	0.000
					q13.2	+0.725196	–	5.38 ^{E-21}
21	NA				q22.3	+1.123901	–	1.88 ^{E-13}
22	NA				NA			

Shown is the average of the log ratio for chromatid gains, amplifications, or deletions in each chromosomal region, indicating the extent of deviation from the normal threshold (0.00). Amplifications are bolded. Note the shared chromatin gain or amplification (underlined) in both tumors in 11p15.5 and 17q21.31-q25.3, including several key genes for WT and NB development.

^a Differences relate to normal human male control using aCGH. p values ≤0.05 were considered significant.
NA = No abnormalities.

[Leone et al., 1993]. *NGFR* (Entrez Gene: 4804; OMIM: 162010) encodes a tumor suppressor protein that is possibly downregulated (silenced) in NB by other genes (*EZH2*) in cases with poor prognosis [Wang et al., 2012]. The product of *AXIN2* (Entrez Gene: 8313; OMIM: 604025) plays an important role in the regulation of the stability of beta-catenin in the Wnt signaling pathway,

and its deregulation is potentially associated with breast cancer, NB, and WT [Mai et al., 1999]. *AATK* (Entrez Gene: 9625; OMIM: 605276) is induced during apoptosis and may be a necessary pre-requisite for the induction of growth arrest and/or apoptosis of myeloid precursor cells. In a NB cell line *AATK* has been shown to produce neuronal differentiation [Raghunath et al., 2000].

Fig. 4. Chromosomal projection of aCGH results from the patient's WT (**A**) and NB (**B**), indicating deletions (green bars) and chromatin gains or amplifications (red bars). Of particular interest are the shared gains or amplifications (circles) in the chromosomal regions 11p15.5 and 17q21.2-q25.3, hot-spot areas for genes associated with WT and NB development.

Other regions with CNV

Chromatid gain 2p25.2-p21 (NB). It affects the genes *MYCN* and *ALK* (Entrez Gene: 4613; OMIM: 164840 and Entrez Gene: 238; OMIM: 105590, respectively) which are intimately associated with the development, pathobiology, and prognosis of NB.

Chromatid gain 7q11.23-q36.3 (NB). It involves the developmental *SHH* gene (Entrez Gene: 6469; OMIM: 600725), member of the hedgehog signaling pathway, whose dysfunction has been implicated in the development of some cancers. Another gene in that region is *WNT2* (Entrez Gene: 7472; OMIM: 147870) which is a member of the Wnt family of signaling proteins implicated in NB oncogenesis [Katoh and Katoh, 2005]. *HGF* (Entrez Gene: 3082; OMIM: 142409) is a multi-functional cytokine with increased serum levels in NB patients with higher stage disease and genetic markers of poorer prognosis [Sköldenberg et al., 2009]. *RELN* (Entrez Gene: 5649; OMIM: 600514) encodes a large extracellular matrix protein thought to control cell-cell interactions critical for cell positioning and neuronal migration during brain development and also plays a role in the development of neural crest derived tumors such as NB [Katyal et al., 2011].

Deletion 17p13.3-p12 (WT). It includes the loss of *TP53* (Entrez Gene: 7157; OMIM: 191170) whose product p53 is an important tumor suppressor (gatekeeper) protein.

The most important aCGH results are summarized in figure 5 with an emphasis on shared variants of potential key players on chromosomes 11p15 and 17q21.

Discussion

It is well established that the prevalence of VL is increased in the most severe subtypes of FA (D1 and N) with high chromosomal and genomic instability and thus high risk of malignancies [Quan and Smith, 1972; Hirsch et al., 2004; Faivre et al., 2005; Reid et al., 2007; DeWire et al., 2009; Kopic et al., 2011]. An early study on the genetic basis of VL focused on the significance of a point mutation (c.3243A>G) in the *MTTL1* gene, a hypothesis later refuted by negative findings of *MTTL1* variants in a cohort of 62 VL patients [Damian et al., 1996; Stone and Biesecker, 1997]. Subsequent studies suggested a potential involvement of the sonic hedgehog (SHH) pathway in the pathogenesis of VL, since the VL phenotype was observed in a knock-in mouse-model with mutant *Gli*, a

Fig. 5. Shared CNV in both tumors in terms of genes. The depicted areas correspond to hot-spots of target genes (genes of interest). NB = neuroblastoma; WT = nephroblastoma (Wilms tumor); + = amplification. Shared potential or established genes of interest in NB and WT are bold and underlined.

gene encoding a transcription factor mediating SHH signal transduction. This finding suggested that defective SHH signaling during embryogenesis might lead to VL and that *Gli*-mutant mice might serve as a VL model [Kim et al., 2001]. Accordingly, a heterozygous 21-bp de novo deletion in a polyalanine tract of the *HOXD13* gene was detected in a 17-year-old girl with anal atresia, tetralogy of Fallot, vesicoureteral reflux, and fusion of the distal interphalangeal joints of the toes, suggesting that the SHH pathway might be involved in the development of gut and genitourinary structures in addition to limb development [Garcia-Barcelo et al., 2008], a combination of organs characteristically affected in VL. However, these observations could not be generalized. A newly described association of VL with the insertion of 6 nucleotides in the GCC repeat of the *ZIC3* gene suggested the involvement of this gene in VL [Chung and Shaffer, 2011], but this likewise remained a unique report. Finally, a termi-

nal deletion of chromosome 7q encompassing the *SHH* gene in a patient with esophageal stenosis implied that haploinsufficiency of *SHH* might have participated in emergence of this VL-like phenotype [Zen et al., 2010]. Our case did not present with findings to corroborate any of these hypotheses.

FA with its associated high chromosomal and genomic instability likely forms the background for the simultaneous occurrence of WT and NB which has been described by several communications [Alter, 2003; Hirsch et al., 2004; Berrebi et al., 2006; Sari et al., 2009; Compostella et al., 2010]. Also the coincidence of WT and NB in patients with VL, probably as a correlate of FA, has been reported previously: one of these patients had numerous congenital malformations (horseshoe kidney, cerebellar hypoplasia, microcephaly, sacral agenesis, and esophageal atresia) and developed simultaneously WT in a horseshoe kidney and bilateral NB at the age of 11 months.

This child later presented with a tumor of the posterior cerebellar fossa and died within a month [Berrebi et al., 2006], a clinical course very similar to our case. Another child with both WT and NB was subsequently diagnosed with FA and died after the first bout of chemotherapy [Compostella et al., 2010]. These latter observations, the above reports on patients with FA and co-occurrence of WT and NB and the further case of an infant with documented FA, medulloblastoma, and WT in a horseshoe kidney suggest an axial predisposition (kidneys–central nervous system) for the development of ‘embryonal’ tumors in FA [de Chadarevian et al., 1985].

The increased risk of ‘embryonal’ cancers of infantile onset is often due to biallelic *BRCA2/FANCD1* mutations which make FA-D1 patients susceptible to the early development of WT, medulloblastoma, hepatoblastoma, acute myelogenous or T-cell leukemia, and primitive neuroendocrine tumors [Hirsch et al., 2004; DeWire et al., 2009; Kopic et al., 2011]. Mutations in *BRCA2/FANCD1* were not detected in our present case, but instead a novel homozygous *PALB2/FANCN* mutation leading to a protein truncation was found. In fact, *PALB2/FANCN* mutations give rise to a very similar spectrum of tumors and age of onset as *BRCA2/FANCD1* mutations. However, further underlying mechanisms such as amplification or deletion of oncogenes or tumor suppressor genes, respectively, must be involved in tumor development in FA patients, since mutations in FA genes were not underlying FA-typical neoplasms such as acute myelogenous leukemia or squamous cell carcinoma of the head/neck or genital region in the general population. Commonly, *TP53* mutations and loss of heterozygosity has been described in FA-associated as in other cancers [Van Zeeburg et al., 2008]. This is consistent with our finding of a deletion in 17p13.3-p12 encompassing the *TP53* gene in WT of the present patient. We also detected several previously reported CNVs associated with WT/NB (with or without FA): the 17q amplification which is the most common unbalanced CNV observed in FA with NB, initially described by Bisig et al. [2002] in association with WT; a partial loss of 14q21ter (common in NB); a gain of 3q22ter and of Xp22.1-p11 (common in WT); and amplifications in genes located at 11p15 (*IGF2*), strongly associated with WT [Royer-Pokora, 2007]. Finally, we observed shared amplifications of key genes in both tumors in chromosome regions with CNV. Among these genes, including *HRAS*, *IGF2*, *CTSD*, and *BIRC5*, are established oncogenes that have been associated with tumor growth, notably WT and NB in children. It cannot be ruled out that overexpression of one of these or other genes from re-

gions with increased copy numbers, in particular those that were common to both neoplasms, contributed to tumorigenesis. In their initial communication on *PALB2* mutations in FA, Reid et al. [2007] reported 3 of 7 patients with dual or even triple malignancies, including various combinations of WT, NB, medulloblastoma, and acute myeloid leukemia. Unfortunately, further studies of tumor DNA in those patients and our present case were restricted due to limited availability of tissue materials.

In conclusion, the analysis of somatic alterations in malignancies of a girl with FA-N and incomplete VL, extreme chromosomal instability, and simultaneous development of WT and NB revealed genomic alterations in terms of CNV, with overlapping amplifications in 11p15.5 and 17q21.31-q25.3 in both tumors, including key genes for WT/NB development. Therefore, this merely descriptive study serves to alert the community to genomic regions of shared amplification among ‘embryonal’ type cancers, in order to incite further observation in more extensive studies and, if confirmed, functional approaches with selected genes to better understand the biology of these tumors.

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Erratum

In the article 'Shared Copy Number Variation in Simultaneous Nephroblastoma and Neuroblastoma due to Fanconi Anemia' by Serra et al. (Mol Syndromol 2012;3:120–130) the name of the ninth author is wrong.
The correct name is B. Royer-Pokora and not B. Pokora.