Original contribution

Loss of PTEN/MMAC1 activity is a rare and late event in the pathogenesis of nephroblastomas☆

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Summary Recent genetic investigations of nephroblastomas point to an activation of the Wnt pathway. Data indicate however that activation might be partly due to cross talk of different signaling pathways including the tumor suppressor gene PTEN (phosphatase and tensin homolog on chromosome 10). Therefore, we examined expression and chromosomal aberrations of PTEN in nephroblastomas of different subtypes and the corresponding nephrogenic rests. Loss of heterozygosity was analyzed by high-resolution melting analysis of 4 different single nucleotide polymorphisms. Results were confirmed by sequence analysis of the polymerase chain reaction products. In addition, an intragenic insertion-deletion polymorphism of the PTEN gene was investigated. Protein expression was assessed by immunohistochemistry. Twenty-two nephroblastomas and their corresponding nephrogenic rests were included in the study. In the high-resolution melting analysis, 15 samples were homozygous, 6 were heterozygous, and for 1 sample results could not be obtained for technical reasons. None of the samples showed loss of heterozygosity. Nineteen of the tumors and corresponding nephrogenic rests were also examined immunohistochemically. All tumors showed cytoplasmic positivity, with the exception of 1 tumor that showed complete loss of staining. In 1 tumor, the epithelial component showed distinct cytoplasmic staining, whereas the immature muscle and hyaline cartilage were negative. All nephrogenic rests exhibited positive cytoplasmic staining of all components. Our results establish that inactivation of PTEN is a rare and late event in the pathogenesis of nephroblastomas.
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1. Introduction

Nephroblastomas are morphologically and genetically complex embryonal renal tumors of childhood. Histologically, nephroblastomas present with 3 cell types differentiating toward blastema, epithelium, and stroma. Each component can be present within a tumor to a varying degree. The diverse cell types may also express a varying degree of differentiation [1].

Nephroblastomas are thought to develop from nephrogenic rests (NRs), their precursor lesions, as subclonal proliferation. NRs are abnormally persistent metanephric remnants of embryonal kidney development. They can be identified in about 1% of children without renal tumors at autopsy. In 30% to 44% of kidneys removed for nephroblastoma, incidental NRs can be found [2]. Classification of NRs is based on their topographic distribution within the renal lobe: perilobar rests can be found subcapsular between the renal pyramids; and intralobar rests, in between the pyramids [3,4]. A more recently developed subclassification takes not only the topographic site into consideration, but also the tumorigenic potential. NRs are therefore classified as obsolete, sclerosing, dormant, hyperplastic, and neoplastic (adenomatous) NRs [1,2]. It is very important to identify incidental NRs, as they harbor an association with bilateral and metachronous contralateral nephroblastomas [5,6].

PTEN is a tumor suppressor gene located on chromosome 10q23.3 influencing cell proliferation, apoptosis, and cellular migration. PTEN is involved in a variety of signal transduction pathways, but also acts via the Akt/PI3K pathway [7]. Inactivation of PTEN has been implicated in the development of a wide variety of malignancies. In several studies, a role of PTEN in the pathogenesis of different types of renal cell carcinomas has been established. Compared with normal renal tissue and oncocytomas, expression of PTEN is significantly reduced or not detectable in renal cell carcinomas [8], also paralleled by increased p-Akt activation [9]. There is strong evidence that the mechanism of inactivation of PTEN in renal cell carcinomas is due to an allelic loss on chromosome 10q23.3 rather than mutations of this gene [10]. Investigations for genomic imbalances with comparative genomic hybridization in nephroblastomas demonstrated loss of chromosomal material at chromosome 10q in some of the cases [11].

The aim of this study was to establish whether inactivation of PTEN gene plays a role during the pathogenesis of nephroblastomas. In addition, we investigated whether changes of PTEN are early or late events during this process by including corresponding NRs of these tumors into our study.

2. Methods

2.1. Samples

All samples were obtained from the children's tumor registry at the Institute of Pediatric Pathology, University of Kiel. Tissue samples had been fixed in 10% buffered formaldehyde solution and embedded in paraffin wax. Sections from paraffin blocks were obtained.

2.2. DNA extraction

Areas of normal tissue, NRs, and nephroblastomas were selected for microdissection after deparaffination and stained with Papanicolaou stain. Areas of interest from tumors and NRs were microdissected manually under microscopic control from 3 to 5 sections, depending on the size of the selected area, as described elsewhere [12]. The microdissected tissue was directly transferred into Eppendorf tubes containing ATL buffer. Genomic DNA was extracted with the QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

2.3. Immunohistochemistry

Sections of 3 μm were deparaffinated followed by pretreatment for 40 minutes in a water bath at 98°C in target retrieval solution at pH 9 (DAKO, Glostrup, Denmark) and a cooling phase for 20 minutes at room temperature. Endogenous peroxidase was blocked with 3% H_2O_2 (DAKO) for 10 minutes at room temperature. PTEN antibodies (Abcam, Cambridge, United Kingdom) were diluted 1:100 in antibody diluent (Dako). Samples were incubated for 30 minutes at room temperature. Dako EnVision Detection System (Dako) was used as detection system incubating slides for 30 minutes at room temperature followed by 10-minute treatment with 3,3′-diaminobenzidine + substrate. Specimens were counterstained with hematoxylin.

2.4. Loss of heterozygosity analysis

2.4.1. High-resolution melting analysis

Primers for high-resolution melting analysis (HRMA) were designed using Primer3 software (http://frodo.wi.mit.edu/) (primers and characterization of single nucleotide polymorphisms in Table 1). Samples were amplified in duplicate 20-μL reactions using the LightCycler 480 High-Resolution Melting Master Kit (Roche Diagnostics GmbH, Vienna, Austria) and analyzed on a LightCycler480 instrument (Roche Diagnostics GmbH). Amplification products were denatured at 95°C for 1 minute, for reassociation cooled down to 40°C for 1 minute, and then melted from 65°C to 95°C with 25 signal acquisitions per degree. Sequence variations were detected using the Gene Scanning Software v1.5 (Roche Diagnostics GmbH).

2.5. Sequencing reaction

Polymerase chain reaction (PCR) products of the HRMA were purified using either SigmaSpin Post-Reaction Purification Columns (Sigma-Aldrich, Vienna, Austria) according to the manufacturer's instructions or Macherey-Nagel (Marchery-Nagel GmbH & Co., Düren, Germany) microtiter plates for amplicons with a length of more than 150 base pairs (bp). Purified PCR products were applied as templates in DNA sequencing reactions using BigDye Terminator v1.1 chemistry (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. After BigDye Terminator removal with SigmaSpin Post-Reaction Clean-

Table 1 Primers and characterization of single nucleotide polymorphisms

Amplicon	Size	Sequences	SNP
1	212 bp	Fw: 5′-TGCAAAAGCCCTTAGACAAGA-3′	rs532678
		Rev: 5′-CCCTTCTCCCTACTTTCCTCA-3′	
2	200 bp	Fw: 5′-AGGCTCCGCTTCAACATCT-3′	rs2735343
		Rev: 5′-TTCTCCAGGATTTCGTCTGC-3′	
3	159 bp	Fw: 5′-GAAATGGCCAGCAGCTACA-3′	rs2248293
		Rev: 5′-AGTGCAATGCCGTAAACCTT-3′	
4	153 bp	Fw: 5′-CACTATTGACAATTTCTCCCCTTT-3′	rs1234213
		Rev: 5′-GCACATATATGCATCCTTGCT-3′	

Abbreviations: SNP, single nucleotide polymorphism; Fw, forward; Rev, reverse.

Up Plates (Sigma-Aldrich), the samples were run on an AB3730 capillary sequencer. Data analysis was performed with SeqScape v2.5 Software (Applied Biosystems).

2.6. Insertion/deletion polymorphism

Amplification of the analytical part of the PTEN gene was performed in a 50-μL PCR with 1 to 2 μL of DNA using the Qiagen Taq PCR kit. The primer pairs were located upstream and downstream of a polymorphic locus with an insertion/deletion polymorphism in the sequence, resulting in a PCR product of either 109 or 105 bp (forward 5′-GAG TCA TCC AGA TTA TCG AGC-3′, reverse 5′-CTT TAT GCA ATA CTT TTT CCT A-3′). After a hot start (94°C, 7 minutes), DNA was amplified for 45 cycles (95°C, 30 seconds; 52°C, 1 minute; 72°C, 1 minute) followed by a final extension at 72°C for 7 minutes. PCR products were checked on a 3% agarose gel for presence of a band at the correct size. Analysis was performed on Spreadex EL 300 mini gel with the Elchrom SEA 2000 (Elchrom Scientific, Cham, Switzerland). Gels were stained with SYBR Gold solution for 20 to 40 minutes at room temperature followed by incubation with destaining solution (Elchrom, DST) for 30 to 45 minutes at room temperature.

3. Results

3.1. Classification of tumor histology

All samples were retrieved from the Children's Tumor Registry at the Department of Paediatric Pathology, Christian-Albrechts University of Kiel, Germany. Twenty-two sporadic nephroblastomas with incidentally diagnosed NRs were included into the study. Two different NRs were identified in 4 samples each. Fourteen nephroblastomas of mixed type were included, 2 stroma rich, 4 blastema rich, and 1 regressive nephroblastoma. The subtype of 1 tumor was not available. One nephroblastoma of mixed type showed diffuse anaplasia. Eighteen perilobar and 8 intralobar NRs were included. According to the new classification, samples consisted of 8 perilobar dormant NRs with multifocal

hyperplasia, 5 intralobar dormant NRs with multifocal hyperplasia, 8 dormant perilobar NRs, 3 dormant intralobar NRs, and 2 hyperplastic perilobar NRs.

3.2. Analysis of loss of heterozygosity

Analysis of loss of heterozygosity (LOH) was performed on 22 different samples of genomic DNA. Results were obtained for 21 samples; 1 PCR product was not available for technical reasons. Fifteen samples were homozygous; 6 samples were heterozygous. None of these samples showed LOH within the HRMA. At the locus of the insertion/deletion polymorphism, persistence of both alleles was identified in all heterozygous tumors investigated. The corresponding intralobar and perilobar dormant NRs revealed no LOH as well. Results from HRMA were confirmed by sequencing analysis of the PCR products (Fig. 1).

3.3. Protein expression

Nineteen nephroblastomas and their corresponding NRs were analyzed immunohistochemically for protein expression of PTEN. Three cases were not available for immunohistochemical investigation. In all tumors except one, cytoplasmic expression of PTEN was identified. We assessed the different morphologic compartments of these tumors in more detail. Fifteen tumors showed cytoplasmic positivity of the epithelial elements. Interestingly, all epithelial elements, including glomeruloid structures, stained positively. In 10 different samples, blastemal elements showed cytoplasmic positivity. Stromal elements with diverse differentiation were represented in 6 different samples. Mesenchymal stroma showed cytoplasmic positivity accentuated in the areas surrounding epithelial elements. In contrast, within the differentiating mesenchymal elements, only mature striated muscle cells revealed cytoplasmic positivity, whereas immature striated muscle cells and hyaline cartilage were negative. The tumor containing these elements showed cytoplasmic positivity in the epithelial component. One tumor showed complete negativity of both the epithelial and blastemal elements.
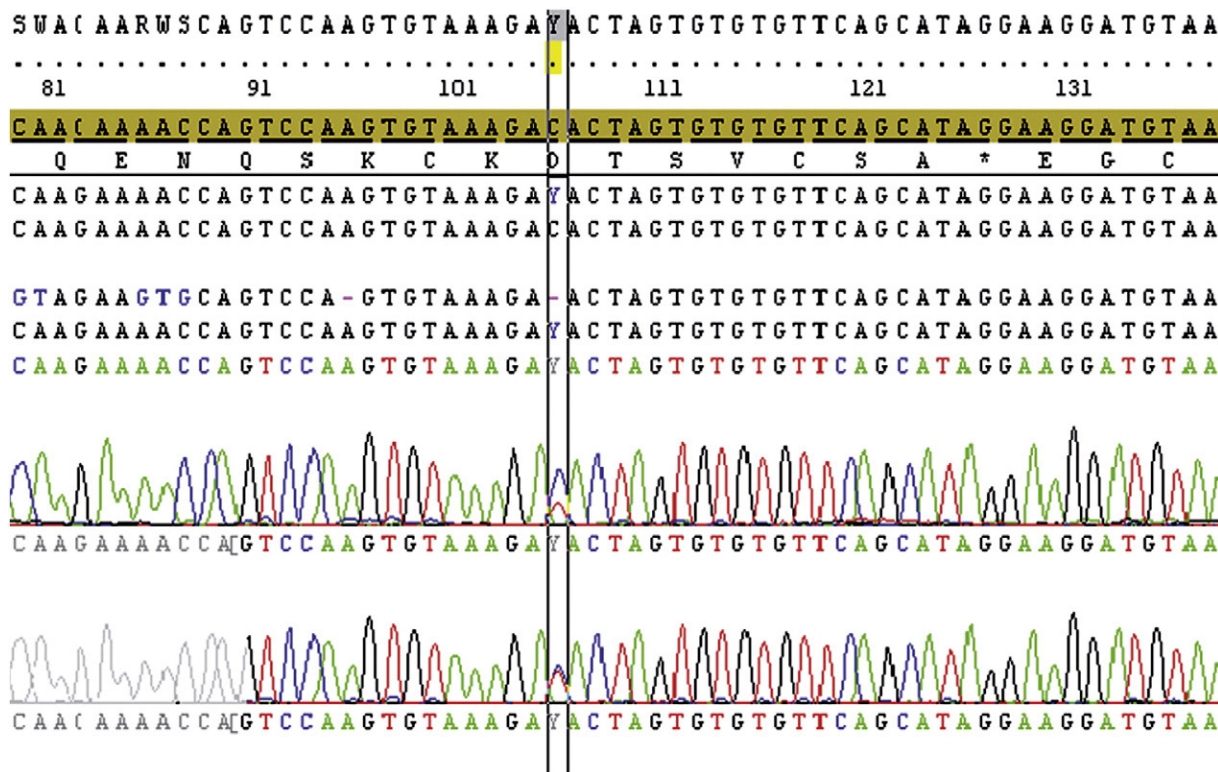
Fig. 1 Sequence analysis of amplicon rs532678. The top sample represents the tumor with complete deficiency of protein expression; the bottom sample represents the corresponding normal tissue. The bars indicate the single nucleotide polymorphism showing 2 peaks in normal and tumor tissue, corresponding to heterozygosity.

Corresponding NRs were all positive in the cytoplasm of all epithelial, blastemal, and stromal elements (Fig. 2).

4. Discussion

Sporadic nephroblastomas show a very heterogeneous molecular pathogenesis. Many candidate genes implicated in the pathogenesis of this tumor have been suggested; but so far, none of these genes appears to play a substantial role in the development of the majority of nephroblastomas. Several studies have attempted to clear the role of β-catenin and activation of the Wnt pathway in the pathogenesis of nephroblastomas. Data from different studies, however, are conflicting. Results are based largely on microarray analysis comparing different types of nephroblastomas and mutational analysis especially of β-catenin and Wilms tumor 1 gene [13,14]. Immunohistochemical analysis of β-catenin however revealed a lack of association of nuclear accumulation of β-catenin with results from mutational analysis of β-catenin [15]. Current investigations of nephroblastomas point to an involvement of the Wnt pathway in the pathogenesis of these tumors by identifying an increase of Wnt pathway regulators in microarray analysis such as TWIST 1 and BCL9, and down-regulation of antagonists such as CTNNBIP1 and CBY1 independent of mutational

status of Wilms tumor 1 and β-catenin [16]. Analysis of different components of nephroblastoma compared with fetal kidney identified an overrepresentation of genes active in the Wnt pathway, among them APC and PLCG2 [17].

These conflicting data point to an alternative genetic activation of the Wnt pathway. Further investigations of the Wnt pathway identified PTEN as one of the candidate genes. Knockdown of β-catenin in a cell line showed involvement and gene expression changes of PTEN and other genes of this signaling pathway [18]. PTEN also affects the Wnt pathway indirectly by controlling the integrin-linked kinase [19].

In our study, we therefore investigated whether changes of the PTEN tumor suppressor gene are involved in the context of the Wnt pathway activation contributing to the development of nephroblastomas. Epithelial and blastemal structures of all but one tumor showed presence of PTEN protein in the cytoplasm. Interestingly, the glomeruloid structures within the epithelial component also stained positively in the cytoplasm, whereas glomeruli of the regular renal parenchyma did not reveal any positivity, as already described in the literature [8]. The mesenchymal stromal component was positive cytoplasmatically; however, differentiated structures showed a diverse pattern of staining. Areas of mature hyaline cartilage were entirely negative for PTEN, which is also known to play a role during bone development influencing skeletal and bone size and
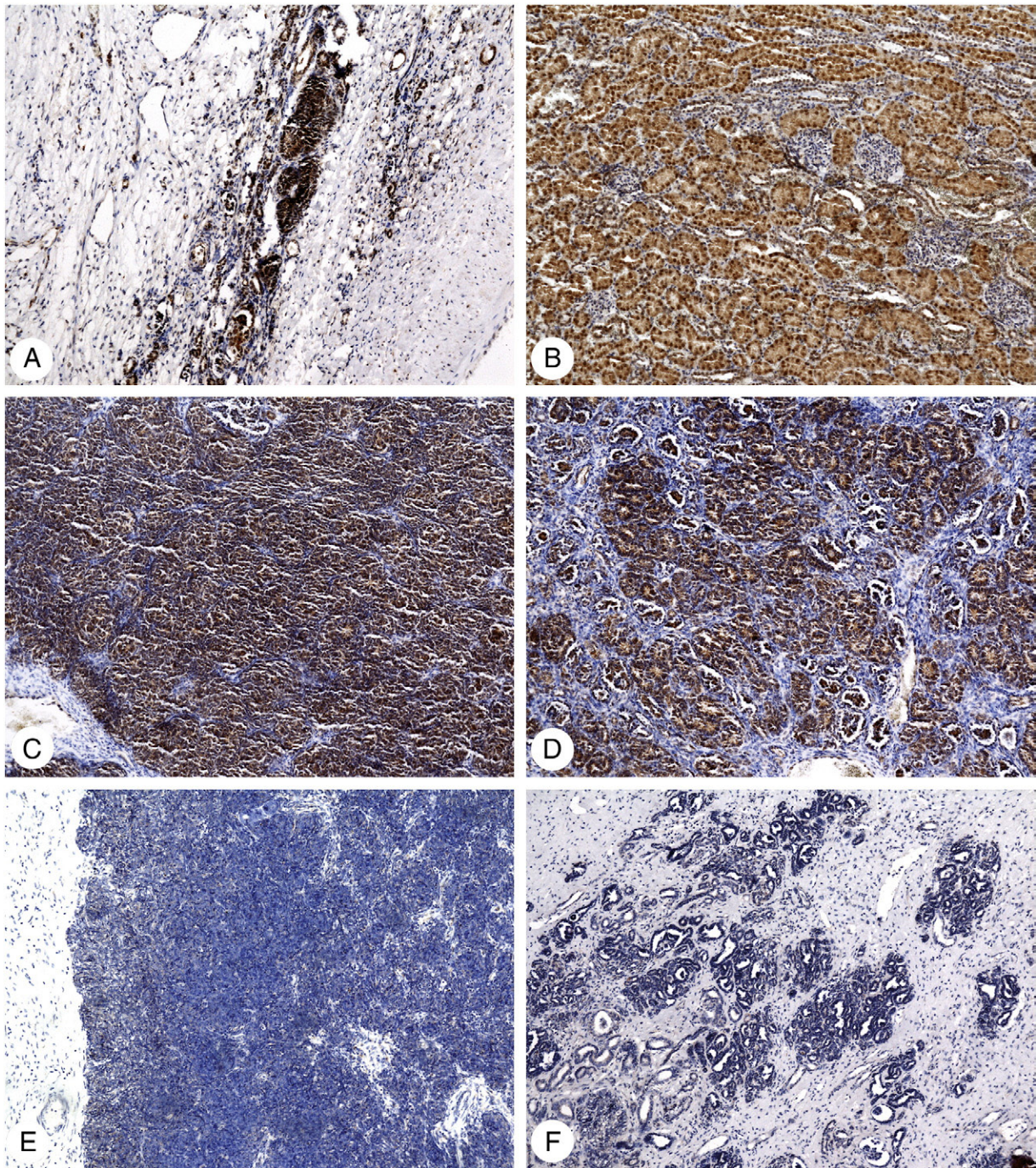
Fig. 2 A, Perilobar dormant NR with hyperplasia showing expression of PTEN. B, Normal kidney parenchyma with negativity of mature glomeruli. C and D, Blastemal and epithelial elements of a mixed nephroblastoma with cytoplasmic PTEN expression including immature glomeruloid structures. E and F, Complete loss of PTEN expression in blastemal and epithelial elements of a mixed nephroblastoma. (A-F: magnification ×100.)

organization of growth plate, but not within the hyaline cartilage [20]. Areas resembling mature skeletal muscle were positive, whereas immature skeletal muscle showed complete negativity. These findings are in keeping with the role of PTEN as inhibitor of protein synthesis with an increased expression with age and maturation of skeletal muscle [21]. Considering all the data, in our opinion, the absence of

staining in immature skeletal muscle and hyaline cartilage reflects probably a developmental process.

LOH rather than mutations in the PTEN gene has been more frequently identified in renal cell carcinomas [10,22]. Therefore, we chose to analyze LOH of PTEN in our samples. None of our samples investigated showed intragenic LOH of the PTEN gene.

Complete loss of PTEN expression was observed in only 1 out of 19 different tumors, epithelial and blastemal structures reacting identically. The corresponding NR showed cytoplasmic positivity. LOH of PTEN was not identified as cause of this observation. The lack of correlation between LOH, mutations, and expression as detected by immunohistochemistry of the PTEN gene has already been well documented [22]. Additional data in the literature suggest that there is progressive loss of PTEN function due to several different mechanisms [23].

Our results indicate that the missing link of alternative activation of the Wnt pathway signaling is not represented by down-regulation of PTEN, as loss of expression of this gene appears to be a rare and late event in the pathogenesis of nephroblastomas.

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