

ANATOMICAL PATHOLOGY

Activation of beta-catenin is a late event in the pathogenesis of nephroblastomas and rarely correlated with genetic changes of the APC gene

CHRISTINE GRILL*¶**, SANDRA SUNITSCH†**, MARTINA HATZ‡, CORNELIA HAUSER-KRONBERGER*, IVO LEUSCHNER§, GERALD HOEFLER† AND BARBARA GUERTL†

*Department of Pathology General Hospital and Paracelsus Medical University Salzburg, Salzburg, †Institute of Pathology, Medical University of Graz, Graz, ‡Center for Medical Research, Medical University of Graz, Graz, Austria; §Kiel Pediatric Tumor Registry, Department of Paediatric Pathology, University Hospital of Schleswig-Holstein, Campus Kiel, Kiel, Germany; ||current address: Institute of Forensic Medicine, Medical University of Graz, Graz, Austria; and ¶current address: Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Iceland, Reykjavik, Iceland: **both authors contributed equally to this work

Summary

Aims: Activation of β -catenin has been identified as a possible mechanism for the development of nephroblastomas. In our study we investigated whether this activation occurs already in precursor lesions of nephroblastomas, called nephrogenic rests (NRs). Inactivation of the adenomatous polyposis coli (APC) protein is an important regulatory mechanism of activating β -catenin. We clarified the role of APC by assessing loss of heterozygosity (LOH) and possible mutations within the genomic region.

Methods: Activation of β -catenin was examined by immunohistochemistry identifying nuclear translocation. Two polymorphic loci of the APC gene were investigated for LOH and sequence analysis was performed for the mutation cluster region of the APC gene on formalin fixed, paraffin embedded samples.

Results: Four of the 18 nephroblastomas available for immunohistochemistry exhibited nuclear staining of β -catenin, but none of the NRs. Analysis of LOH revealed 14 homozygous samples, 10 heterozygous tumours and six tumours exhibiting LOH of the APC gene. One blastema-type nephroblastoma showed nuclear localisation of β -catenin in conjunction with LOH of the APC gene. Analysis of 12 nephroblastomas revealed no sequence aberration.

Conclusion: Our results indicate that nuclear activation of β -catenin is a late event in the tumorigenesis of nephroblastomas coinciding in some tumours with LOH of the APC gene.

Key words: β -catenin, APC, immunohistochemistry, LOH, mutation analysis, nephroblastoma, nephrogenic rest.

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INTRODUCTION

Nephroblastomas are embryonal tumours of the kidney occurring during childhood. Morphologically, nephroblastomas resemble developing kidney and present with three types of tissue forming: blastema, epithelium and stroma. Nephroblastomas can contain a variable amount of each component. The tissue types may also show different degrees of morphological differentiation.¹

Nephroblastomas are considered to develop from nephrogenic rests (NRs), their presumed precursor lesions. NRs are abnormally persistent metanephric remnants of embryonal renal development beyond 36 weeks of gestation. At autopsy, NRs can be found in about 1% of children without renal tumours. Incidental NRs can be identified in 30–44% of kidneys removed for nephroblastoma.² Classification of NRs is principally based on their topographic position in relation to the renal lobe: perilobar rests are located subcapsular and intralobar rests in between the pyramids.^{3,4} A second subclassification additionally reflects their oncogenic potential. Accordingly, NRs are subdivided into obsolete, sclerosing, dormant, hyperplastic and neoplastic (adenomatous) NRs.^{1,2} Identification of incidental NRs is very important, as these lesions have been associated with bilateral and metachronous contralateral nephroblastomas.^{5,6}

The molecular pathogenesis of sporadic nephroblastomas appears to be very heterogenous. A variety of genes have already been described as being involved, implicating multiple cellular signalling pathways. Investigated in most detail thus far is the classical canonical Wnt pathway with β -catenin as one of the key regulators.⁷ Genetic aberrations of β -catenin in nephroblastomas have been examined in several studies, but nuclear translocation did not always coincide with stabilising mutations of β -catenin. In a majority of cases, nuclear translocation was only verified in 5–10% of tumour cells, mostly in the undifferentiated mesenchyme surrounding epithelial tubules.^{8–10} Some studies could not find any nuclear translocation in nephroblastomas.¹¹ Activation of β -catenin in NRs has so far only been explored in one study analysing two cases of nephroblastoma and corresponding NRs. This investigation revealed mutations of β -catenin in the tumour, but not in the NRs. Noteworthy, immunohistochemistry revealed positive nuclear staining only in the stromal areas of nephroblastoma with rhabdomyogenic differentiation.¹²

One important regulatory mechanism of β -catenin is inactivation in a cytoplasmic destruction complex containing several proteins.^{13,14} Adenomatous polyposis coli (APC) protein is part of this destruction complex and acts as a classical tumour suppressor gene. One important mechanism for inactivation of this gene is loss of heterozygosity (LOH).¹⁵ So far, however, little is known about the role of the APC gene in the

pathogenesis of nephroblastomas. In one study up to 30% of nephroblastomas showed allelic loss or allelic imbalances at a polymorphic microsatellite locus on chromosome 5q, involving either the APC gene or other tumour suppressor genes.¹¹

In our study we wanted to investigate systematically whether activation of β -catenin is an early or late event in the pathogenesis of nephroblastomas. Furthermore, we determined whether genetic aberrations of the APC gene play a role in the activation of β -catenin.

MATERIAL AND METHODS

Samples

Thirty-six nephroblastomas and 10 corresponding NRs were included in the study. Tissue samples had been fixed in 4% buffered formaldehyde solution, and embedded in paraffin wax. Sections were cut at 3 μ m thickness.

Immunohistochemistry

Sections were deparaffinised followed by pretreatment for 40 min in a water bath at 98°C in target retrieval solution pH9 (Dako, Denmark) and a cooling phase for 20 min at room temperature (RT). Endogenous peroxidase was blocked with 3% H₂O₂ (Dako) for 10 min at RT. Two different antibodies were used for detection of β -catenin, in order to obtain representative results, as different staining patterns have been described for diverse antibodies in the literature.^{8–11} The antibodies were diluted 1:200 (Santa Cruz Biotechnology, USA; Cat. No. SC-1496) and 1:600 (BD Transduction Laboratories, USA; Cat. No. 610154) respectively, in antibody diluent (Dako). Samples were incubated for 30 min at RT. The Dako EnVision Detection System (Dako) was used as the detection system, incubating slides for 30 min at RT followed by 10 min treatment with 3,3'-diaminobenzidine (DAB) + substrate. Specimens were counterstained with haematoxylin.

DNA extraction

Areas of normal tissue and nephroblastoma were selected after deparaffinisation, stained with Papanicolaous stain and dissected manually under microscopic control into 3–5 sections, depending on the size of the selected area.¹⁶ The dissected tissue was transferred directly into a buffer of the QIAamp DNA mini kit (Qiagen, Germany). Genomic DNA was extracted using the QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions.

Analysis of LOH

Amplification of the analytical part of the APC gene was performed in a 25 μ L polymerase chain reaction (PCR) with 50 ng of genomic DNA using the Qiagen Hot Start Taq DNA-polymerase System (Qiagen).

Polymorphic locus 1458

Primer pairs were located upstream and downstream of a highly polymorphic locus containing T or C in the sequence, resulting in a PCR product of 215 bp (for primer sequences see Table 1). After a hot start (95°C, 15 min) DNA was amplified for 40 cycles (95°C, 20 s; 55°C, 25 s; 60°C, 2 min) followed by a final extension at 72°C for 10 min. A representative fraction of 5–10 μ L of the PCR product was treated for 4 h with the restriction endonuclease RsaI (New England Biolabs, USA) followed by inactivation of the enzyme at 95°C for 3 min. In the presence of a C the PCR product was cut into two fragments of 130 bp and 85 bp

Table 1 PCR primer pairs for analysis of LOH of the APC gene

Primer pair PCR 1458	1458.5	5'GATGATTGTCTTTTTCCTTGC3'
	1458.3	5'CTGAGCTATCTTAAGAAATACATG3'
Primer pair PCR 4520	4520–1	5'TACCTAAAAATAAGCACCTAC3'
	4520–2	5' GATTCTGTTTCATTCCATCGTCA3'

in length.¹⁷ The digested PCR products were analysed on a 3% agarose gel stained with ethidium bromide.

Polymorphic locus 4520

Primer pairs were located upstream and downstream of the polymorphic locus containing A or G in the sequence, resulting in a PCR product of 304 bp as described elsewhere¹⁸ (for primer sequences see Table 1). After a hot start (95°C, 15 min) DNA was amplified for 40 cycles (95°C, 20 s; 55°C, 25 s; 60°C, 2 min) followed by a final extension at 72°C for 10 min. A representative fraction of 5–10 μ L of the PCR product was incubated for 4 h with the restriction endonuclease BtgI (New England Biolabs) exhibiting the same sequence specificity as BsaI, followed by inactivation of the enzyme at 95°C for 3 min. In the presence of A the PCR product was cut into two fragments of 169 bp and 135 bp in length.¹⁹ The digested PCR products were analysed on a 3% agarose gel stained with ethidium bromide.

Sequencing

PCR products were purified using SigmaSpin Post-Reaction Purification columns (Sigma-Aldrich, USA) according to the manufacturer's instructions. The purified PCR products were used as templates in DNA sequencing reactions using BigDye Terminator v1.1 chemistry (Applied Biosystems, USA). Primer sequences were located in the mutation cluster region of the APC gene (see Table 2). The samples were run on an AB3730 capillary sequencer and data analysis was performed with SeqScape Software (Applied Biosystems). Sequencing reactions also included analysis of the single nucleotide polymorphism (SNP) rs4115.

RESULTS

Classification of tumour histology

Thirty-six sporadic nephroblastomas were included in the study, 13 of mixed type, two of stroma-type, four of blastema-type, one of epithelial-type, 11 regressive nephroblastomas and one partially cystic differentiated nephroblastoma. The subtype of four tumours was not available. All samples of regressive nephroblastomas contained viable tumour areas.

Immunohistochemistry

Samples of 18 nephroblastomas containing large areas of viable tumour and 10 corresponding NRs were available for immunohistochemical investigation. All NRs analysed, including six perilobar NRs and four intralobar NRs, had cytoplasmic and membranous or focal membranous staining in tubular

Table 2 Primer pairs for sequencing of the mutation hotspot region of the APC gene

Primer pair APC_Ex16a	APC_Ex16a-fw APC_Ex16a-rev	5'GGCTGCCACTTGCAAAGT3' 5'AACTAGAACCCTGCAGTCTGC3'
Primer pair APC_Ex16b	APC_Ex16b-fw APC_Ex16b-rev	5'AGAACCAAATCCAGCAGACTG3' 5'CTTGGTGGCATGGTTTGTGC3'
Primer pair APC_Ex16b-2	APC_Ex16b-fw2 APC_Ex16b-rev2	5'GTGAGCGAAGTTCAGCAG3' 5'GCACCACTTTTGGAGGGA3'
Primer pair APC_Ex16c	APC_Ex16c-fw APC_Ex16c-rev	5'CATTATAAGCCCCAGTGATCTTC3' 5'ATAAATGGCTCATCGAGGCT3'
Primer pair APC_Ex16d	APC_Ex16d-fw APC_Ex16d-rev	5'CCAGATGGATTTTCTTGTTCAT3' 5'TGTTGGCATGGCAGAAA3'

structures and surrounding mesenchyme (see Fig. 1). However, no nuclear staining was detected in any of the NRs.

All tumours, including nine mixed nephroblastomas, two blastema-type, five regressive and two stroma-type nephroblastomas, showed expression of β -catenin differing in staining pattern and distribution. Four of the 18 nephroblastomas analysed exhibited nuclear staining. One of these tumours was positive in the blastema. One showed positivity in the blastema, in very immature glomeruloid bodies and focally in the undifferentiated stroma surrounding tubules. One of the tumours was focally positive in undifferentiated mesenchymal cells surrounding immature tubules. One stroma-rich nephroblastoma showed diffuse cytoplasmic and nuclear positivity in mesenchymal areas with skeletal muscle differentiation and only cytoplasmic and membranous positivity in the epithelial component. In one sample nuclear expression was noted in epithelial structures differentiated as immature glomeruloid bodies.

Detailed analysis of the different morphological areas within the tumours without nuclear staining was performed. Ten areas

with blastemal differentiation and 14 areas with epithelial differentiation stained within the cytoplasm and at the cell membrane. Seven areas of glomeruloid differentiation were completely negative. Five stromal areas stained at least in part positive in the cytoplasm and at the membrane and five were negative including hyaline cartilage and immature skeletal muscle (Fig. 1).

The two antibodies against β -catenin used in our study showed different results, as already described in the literature for antibodies against β -catenin.⁸⁻¹¹

Analysis of LOH

In 33 of 36 samples an amplification of the extracted DNA could be performed. Thirteen samples of non-tumoural corresponding tissue for the tumours were homozygous, therefore an analysis of these tumours would not have been informative.

Twenty samples revealed heterozygosity, however for five corresponding tumours PCR products could not be obtained for technical reasons. Nine samples showed heterozygosity for the

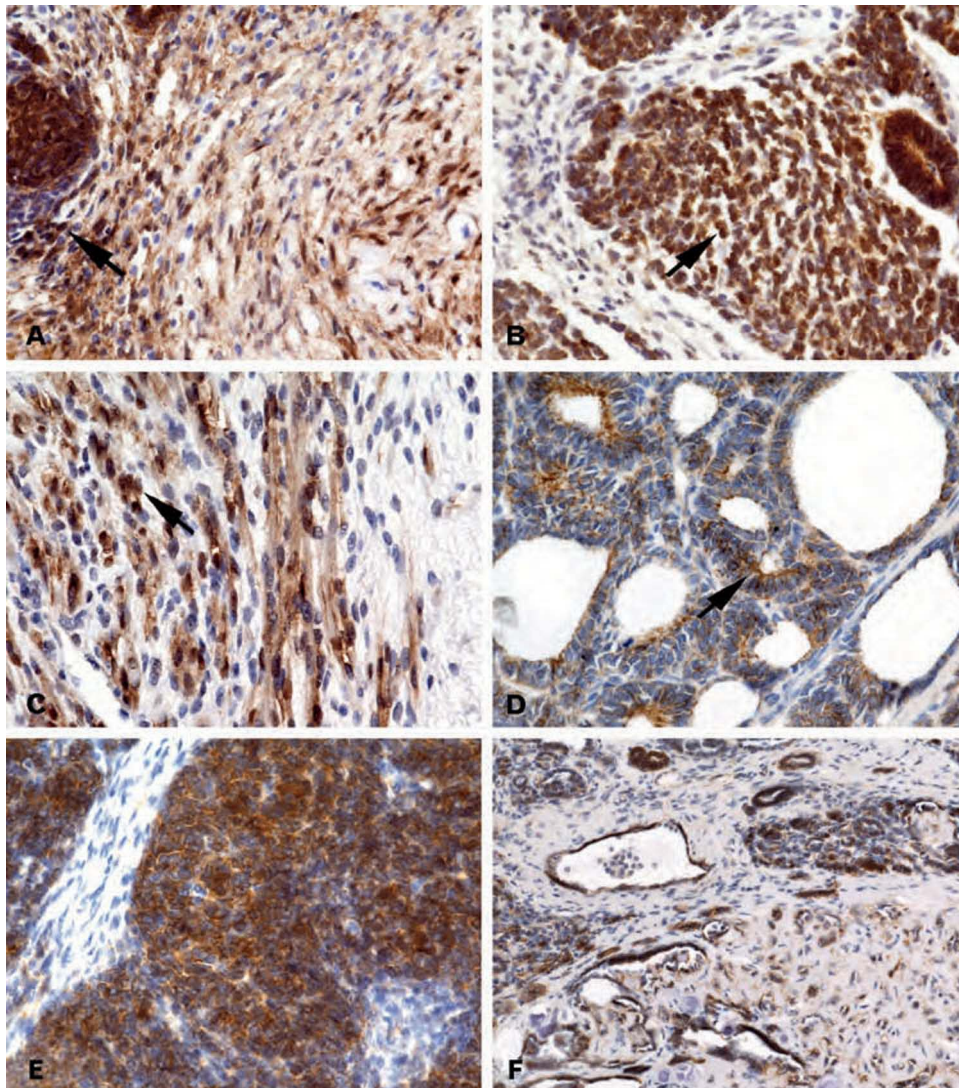


Fig. 1 (A) Immunohistochemistry of β -catenin shows focal stromal nuclear positivity in the mesenchymal areas surrounding epithelial structures, indicated by an arrow. (B) Nuclear positivity of β -catenin is also detected in blastema and (C) in the ectopic mesenchymal component differentiated into skeletal muscle together with cytoplasmic and membranous staining. (D) Strong cytoplasmic and membranous positivity is found in epithelial structures and (E) blastema. (F) All NRs have membranous and cytoplasmic expression of β -catenin.

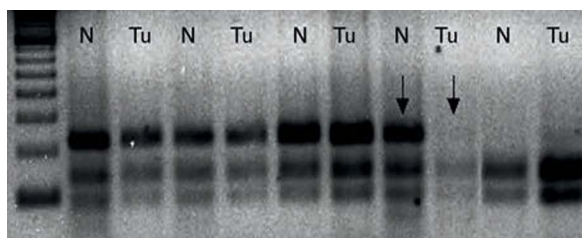


Fig. 2 Analysis of LOH of the APC gene at the polymorphic locus 1458. Arrows indicate heterozygous normal tissue (N) with bands at 215 bp, 130 bp and 85 bp after restriction enzyme digestion and LOH in the corresponding nephroblastoma (Tu) as indicated by the loss of the top lane (loss of the T-allele) at 215 bp.

tumours; in six samples LOH was detected in the tumour tissue (Fig. 2).

Sequencing

The hot mutation cluster region of the APC gene of 12 tumour samples was sequenced to identify possible mutations. None of the samples investigated showed any aberrations of the sequence, although three of the tumours harboured LOH of the APC gene.

Sequencing analysis of the SNP rs4115 gave additional information for two tumour samples, for which analysis of LOH had not been possible by restriction enzyme digestion. One tumour was heterozygous and one homozygous (Fig. 3).

DISCUSSION

Activation of the classical canonical Wnt pathway has been regarded as an important event in the pathogenesis of sporadic nephroblastomas and has also been correlated with chromosomal aberrations of WT1 and β -catenin, as well as

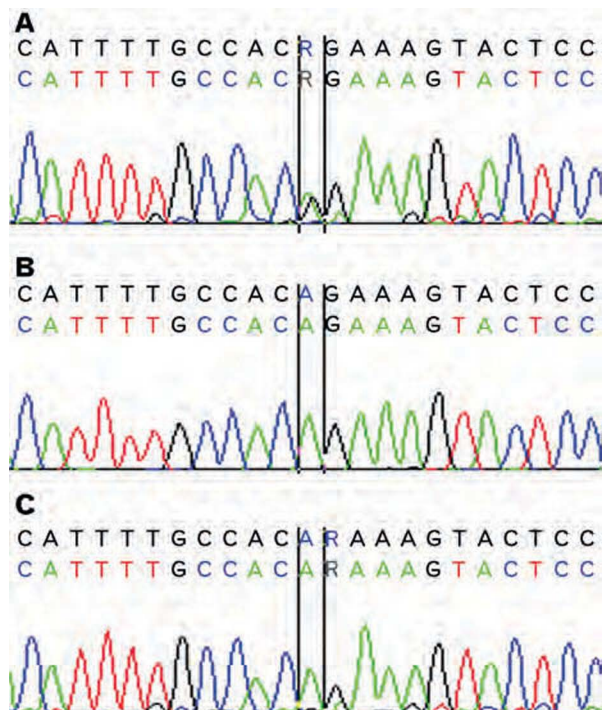


Fig. 3 (A) Sample of a heterozygous tumour indicated by two peaks in the sequence analysis. (B) A homozygous sample indicated by one peak in the tumour sample and (C) in the corresponding normal sample.

nephroblastomas containing ectopic mesenchymal elements.^{9,10} Recent results from microarray analysis of nephroblastomas, however, point to an involvement of the Wnt pathway independent of mutational status of WT1 and β -catenin as well as the morphological pattern of the tumour.^{10,20} In our study we investigated the importance of the activation of β -catenin in nephroblastomas and their presumed precursor lesions, NRs, identified by nuclear translocation of this transcription factor. None of the NRs investigated harboured nuclear translocation of β -catenin, regardless of the subtype of NR and the results within the corresponding tumour. Four of the 18 tumours investigated exhibited nuclear staining of β -catenin. In one area of extremely immature glomeruloid structures, nuclear staining of β -catenin was identified. This distinct staining pattern seems to reflect a developmental process of kidney organogenesis, as developing kidneys of newborn rats showed significant staining in the capillary loop stage. This expression disappeared during the maturation of glomeruli.²¹

One of the stroma-type nephroblastomas harboured a large amount of ectopic mesenchymal elements, differentiated as striated muscle. Within this area, diffuse strong cytoplasmic and nuclear staining were detected. Similar results have already been described in the literature with strong nuclear staining of skeletal muscle elements within nephroblastomas, regardless of the mutational status of β -catenin. However, not all of the tumours followed this pattern and some myogenic elements showed no β -catenin staining.²² In our own series we also identified one tumour with immature myogenic elements without nuclear β -catenin staining.

Two of the tumours showed positive nuclear staining of β -catenin in the undifferentiated mesenchyme surrounding tubules lined by epithelial cells, in agreement with results already reported in the literature.⁸ Nuclear localisation of β -catenin in epithelial tumours is regarded as aberrant expression and as a sign of epithelial-mesenchymal transition (EMT) only present in cancer cells,²³ whereas nuclear localisation of β -catenin in mesenchymal cells might be necessary to maintain the undifferentiated mesenchymal state.²⁴ Similar results can be identified in the developing kidney, where β -catenin is one of the main triggers in epithelial conversion from mesenchymal progenitors.²⁵ Therefore, these areas of nuclear staining might indicate the process of mesenchymal-epithelial transition (MET) in nephroblastomas, reiterating kidney development rather than an oncogenic activation. One mixed nephroblastoma and an additional blastema-type tumour showed diffuse nuclear positivity of β -catenin implying oncogenic characteristics, as already described in other malignant tumour entities.^{26,27}

Regulation of β -catenin activity is a very complex process involving multiple proteins and protein complexes. In the absence of Wnt ligands β -catenin is usually degraded by ubiquitination bound to a complex containing APC amongst other proteins. If this complex cannot bind β -catenin or if a ligand binds, β -catenin is stabilised in a hypophosphorylated state and shows preferential nuclear localisation.⁷ So far, however, little is known about the role of the APC gene in the activation of β -catenin in nephroblastomas. Only one study was performed using microsatellite markers to indicate genetic changes at chromosome 5q, but without further analysis of other genes in this area. Up to 30% of the cases showed allelic imbalance or allelic loss. The authors presume from these results that other tumour suppressor genes at these loci rather

than the APC gene itself might be implicated in tumorigenesis.¹¹ In contrast to the study of Ramburan *et al.*, we investigated polymorphic loci within the genomic region of the APC gene for evidence of a possible LOH, as LOH of the APC gene has been demonstrated to be one important mechanism for loss of function of this tumour suppressor gene.¹⁵ In six nephroblastomas out of 16 informative cases, LOH of the APC gene was identified.

Another very important mechanism of inactivation of the APC gene is mutations, especially truncating mutations.¹⁵ Therefore, we sequenced the mutation cluster region of 12 different nephroblastomas; however, no sequence aberration was identified.

One blastema-type nephroblastoma with LOH of the APC gene showed nuclear activation of β -catenin within the blastema. In contrast, three of the nephroblastomas harboring LOH of the APC gene had only cytoplasmic and membranous β -catenin staining.

Therefore, nuclear activation of β -catenin appears to be a late event in the tumorigenesis of nephroblastomas and is rarely correlated with LOH of the APC gene. Our results indicate that activation of β -catenin might be triggered in most cases by mechanisms other than inactivation of the APC gene, as LOH and mutations are the most important and most commonly observed mechanisms of inactivation for this tumour suppressor gene.

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Address for correspondence: Dr B. Guertl, Institute of Pathology, Medical University of Graz, Auenbruggerplatz 25, A-8036 Graz. E-mail: barbara.guertl-lackner@medunigraz.at

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