Microsatellite analysis of the adenomatous polyposis coli (APC) gene and immunoexpression of β catenin in nephroblastoma: a study including 83 cases treated with preoperative chemotherapy

A Ramburan, F Oladiran, C Smith, G P Hadley, D Govender

Aims: To determine whether microsatellite mutations of the adenomatous polyposis coli (APC) gene have pathological or prognostic significance in nephroblastosomas and to correlate APC alterations with β catenin immunoexpression.

Methods: One hundred nephroblastosomas were analysed, 83 of which received preoperative chemotherapy. Normal and tumour DNA was isolated using standard proteinase K digestion and phenol/chloroform extraction from paraffin wax embedded tissue. Polymerase chain reaction using four APC microsatellite markers—D5S210, D5S299, D5S82, and D5S346—was performed and the products analysed. Immunohistochemistry was performed using the LSAB kit with diaminobenzidine as chromogen. Results were correlated with clinicopathological data using the $\chi^2$ test.

Results: Allelic imbalance/loss of heterozygosity was more frequent than microsatellite instability, with 30% of cases showing allelic imbalance/loss of heterozygosity and 16% showing microsatellite instability. Although there was a significant correlation between the results for individual markers and the clinicopathological data, the overall results do not support a prognostic role for APC in nephroblastoma. Expression of β catenin was seen in 93% of cases. Staining was predominantly membranous, with epithelium, blastema, and stroma being immunoreactive. Cytoplasmic redistribution was seen in 58% of cases, but no nuclear staining was detected. No significant associations between β catenin expression and the clinicopathological parameters were found. Kaplan–Meier survival plots showed that patients with loss of membranous staining and pronounced cytoplasmic staining (score, 3) had a significantly shorter survival ($p=0.04$; median survival, 5.87 months).

Conclusion: Microsatellite analysis of APC and immunoexpression of β catenin did not provide significant pathological or prognostic information in this cohort of nephroblastosomas.

The tumour suppressor adenomatous polyposis coli (APC) gene is frequently mutated or deleted in colon cancers. Germ line mutations in the APC gene are responsible for the autosomal dominant inherited disease familial adenomatous polyposis, whereas somatic mutations in APC occur in approximately 80% of sporadic colorectal tumours. The APC gene is a relatively large gene, which encodes a 8.5 kb mRNA molecule and a 2843 amino acid protein in its most common isoform. The APC protein is structurally complex, with many possible protein–protein interaction sites, including binding sites for β catenin, EBI, and axin. APC participates in a variety of cellular functions including proliferation, differentiation, apoptosis, adhesion, migration, and chromosomal segregation.

The APC gene product is part of a multiprotein complex that regulates the cytoplasmic concentration of β catenin, which has two major functions, cell adhesion and the transmission of the proliferation signal of the Wnt pathway. Under normal circumstances, APC binds directly to free β catenin and promotes its phosphorylation by glycogen synthetase kinase 3β (GSK-3β), thereby targeting β catenin for degradation by the proteasome system. However, when there are mutations in the APC or β catenin gene, especially in the GSK-3β phosphorylation region, APC fails to bind β catenin, which then accumulates within the cell cytoplasm and nucleus. Within the nucleus, β catenin binds directly to the T cell factor/lymphoid enhancer factor group of DNA binding transcription factors and stimulates the transcription of target genes. C-myc, cyclin D1, and fibronectin have been identified as possible target genes for the APC–β catenin pathway. Overexpression of c-myc and/or cyclin D1 is frequently seen in various human cancers, and fibronectin plays an important role in cancer metastasis.

"APC participates in a variety of cellular functions including proliferation, differentiation, apoptosis, adhesion, migration, and chromosomal segregation"
nephroblastoma, whereas losses at loci 11q, 7p, 4q, 3q, 9p, 17p, and 16q have been associated with an outcome or phenotype, but have no direct role in tumour development.21–26

Based on findings from our microsatellite study on the deleted in colorectal cancer gene (DCC), where multiple microsatellite aberrations of this tumour suppressor gene conferred a worse prognosis,27 we extended the study to evaluate allelic imbalance/loss of heterozygosity and microsatellite instability of the APC gene. Therefore, our present study was conducted to determine whether mutations in the microsatellite sequences of the APC gene have pathological or prognostic significance in nephroblastomas. Furthermore, the APC alterations were correlated with β catenin immunoexpression.

MATERIALS AND METHODS

Our study comprised 100 cases of nephroblastoma from the files of the department of pathology, University of KwaZulu-Natal, Durban, South Africa. All cases were obtained from nephrectomies performed over an 18 year period from 1984 to 2001. Clinical data were available for all patients. Patients were managed according to the protocols of the International Society of Paediatric Oncology. Preoperative chemotherapy was administered in 83 of the 100 patients studied. Haematoxylin and eosin stained slides were reviewed and the histological type and tumour staging parameters were confirmed. In our study, nephroblastomas with unfavourable histology referred to those nephroblastomas with diffuse anaplasia.28 Normal DNA was extracted from normal renal tissue (n = 97) and normal lymph nodes (n = 3). For the negative control the primary antibody was replaced by phosphate buffered saline in the staining method.

Microsatellite study

The standard proteinase K digestion and phenol/chloroform extraction method was used for DNA extraction. Amplifiable DNA was verified by polymerase chain reaction (PCR) demonstration of the insulin gene.28

Primer sequences were obtained from the Genome Database (http://www.gdb.org). Table 1 lists the Cy5 labelled microsatellite primers (Roche Diagnostics, Mannheim, Germany) used to amplify the APC gene. These primers were chosen for their high informativity rate.

PCR was carried out in a total volume of 25 μl, containing 10pmol of each Cy5 labelled primer pair, 2 μl template DNA, 200μM dNTPs, 50mM PCR buffer containing 1.5mM MgCl₂, and 0.75 U Taq DNA polymerase. The PCR amplification was performed using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, California, USA). The PCR consisted of an initial denaturation step of five minutes at 95°C, followed by 35 cycles of one minute at 94°C, 30 seconds at 55°C, and 30 seconds at 72°C, with a final extension step at 72°C for seven minutes. Aliquots of 3 μl of the microsatellite PCR products were mixed with 3 μl of STOP solution (blue dextran 2000, deionised formamide; Pharmacia Biotech, Uppsala, Sweden) and denatured at 96°C for three minutes in a GeneAmp PCR System 9700 (Applied Biosystems). The samples were held on ice before being loaded on to the gel. A Cy5 labelled 50–500 bp standard was used as the external size marker (Pharmacia Biotech).

The microsatellite PCR products were analysed on the ALF Express automated DNA sequencer using a 6% Longranger sequencing gel (FMC Bioproducts, Rockland, Maine, USA). The analysis was performed under the following conditions: 1500 V, 60 mA, 15 W, at a constant temperature of 55°C. The data were analysed using the Fragment Manager software program.

Allelic imbalance was determined by using the following ratio: (T2 × N1)/(T1 × N2), where T/N 1 and 2 are the first and second peaks of tumour and normal DNA, respectively.29 A ratio < 0.6 was regarded as allelic imbalance with loss of the larger allele, and a ratio > 1.67 was regarded as loss of the smaller allele. The presence of a novel allele in the tumour sample was interpreted as microsatellite instability.29

Immunohistochemical study

The β catenin specific antibody (clone CAT-5H10; Zymed Laboratories Inc, San Francisco, California, USA) was used at a dilution of 1/300. Tissue sections (2 μm) were picked up on poly-L-lysine (Sigma Diagnostics, St Louis, Missouri, USA) coated slides and routinely dewaxed in xylene and alcohol. To achieve optimal antigen retrieval, the sections were incubated in a microwave oven (H2500 Microwave Processor; Energy Beam Sciences, Agawam, Massachusetts, USA) for 10 minutes at 85°C in 0.01 mol/litre trisodium citrate buffer at pH 6.0. The slides were incubated in 3% H₂O₂ for five minutes to quench endogenous peroxidase activity. The peroxidase labelled streptavidin biotin kit (Dako) was used to perform the tests and 3,3 diaminobenzidine (liquid DAB; Dako) was used as a chromogen. Sections were then counterstained with Mayer’s haematoxylin. Positive (normal breast tissue) and negative controls were run simultaneously. For the negative control the primary antibody was replaced by phosphate buffered saline in the staining method.

Table 1 Chromosome 5q markers

<table>
<thead>
<tr>
<th>Marker</th>
<th>Primer sequence</th>
<th>Chromosome location</th>
<th>Map position (cM)</th>
<th>Heterozygosity</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSS582</td>
<td>(F) ATCCAGGTATCAGAATTC</td>
<td>5q14–q21</td>
<td>118.8</td>
<td>0.758</td>
<td>169–179</td>
</tr>
<tr>
<td>DSS299</td>
<td>(F) GCTACTCTCAGGACTGCT</td>
<td>5q15–q22</td>
<td>127.0</td>
<td>0.704</td>
<td>156–182</td>
</tr>
<tr>
<td>DSS346</td>
<td>(F) ACTCACCTTCCAGTAAAATCGGG</td>
<td>5q21–q22</td>
<td>128.9</td>
<td>0.820</td>
<td>96–122</td>
</tr>
<tr>
<td>DSS210</td>
<td>(F) AATATGGAGGGACTGGCC</td>
<td>5q31.3–q33.3</td>
<td>169.1</td>
<td>0.782</td>
<td>112–132</td>
</tr>
</tbody>
</table>

F, forward; R, reverse.
The immunostaining was graded according to the method of Jass et al. Briefly, a score of 1 was awarded for loss of cell membrane staining; 1 or 2 for slight and pronounced increase in cytoplasmic staining, respectively; and 1 or 2 for slight and pronounced nuclear staining, respectively. The maximum total score was 5 and cases scoring 4 or more were regarded as positive for abnormal β-catenin immunolocalisation. The grading was performed manually (DG, AR) using an Olympus BX40 microscope.

### Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences software program (SPSS, Chicago, Illinois, USA). The results of APC microsatellite...
Table 4  Clinicopathological stage versus marker DSS82 (informative cases)

<table>
<thead>
<tr>
<th>Marker</th>
<th>Stage 1</th>
<th>Stage 2</th>
<th>Stage 3</th>
<th>Stage 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>No change, heterozygous</td>
<td>1</td>
<td>13</td>
<td>9</td>
<td>12</td>
</tr>
<tr>
<td>Allelic imbalance/LOH</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Microsatellite instability</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>p Value</td>
<td>0.011</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5  Patient outcome versus marker DSS82 (informative cases)

<table>
<thead>
<tr>
<th>Marker</th>
<th>Alive</th>
<th>Dead (TR)</th>
<th>Dead (NTR)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No change, heterozygous*</td>
<td>17</td>
<td>16</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Allelic imbalance/LOH</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Microsatellite instability</td>
<td>0</td>
<td>8</td>
<td>0</td>
<td>0.027</td>
</tr>
</tbody>
</table>

Table 6  Results of the APC markers versus administration of preoperative chemotherapy (informative cases)

<table>
<thead>
<tr>
<th>Marker</th>
<th>No</th>
<th>Yes</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSS82</td>
<td>1</td>
<td>4</td>
<td>0.902</td>
</tr>
<tr>
<td>DSS210</td>
<td>1</td>
<td>6</td>
<td>0.078</td>
</tr>
<tr>
<td>DSS299</td>
<td>1</td>
<td>10</td>
<td>0.713</td>
</tr>
<tr>
<td>DSS346</td>
<td>1</td>
<td>16</td>
<td>0.362</td>
</tr>
</tbody>
</table>

RESULTS

There were 51 female and 49 male patients. The patients’ ages ranged from 2 months to 14 years. The median age at diagnosis was 3 years. Eighty three patients received preoperative chemotherapy. There were 83 favourable histology and 17 unfavourable histology (diffuse anaplasia) tumours. Seventeen patients had stage I disease, 23 had stage II, 23 had stage III, and 40 had stage IV disease. There were two stage V patients who were further substaged as stage I (both kidneys) and stage IV. Twenty eight patients were alive and disease free. There were 53 tumour related deaths, two treatment related deaths, and four deaths as a result of other illnesses. Thirteen patients were lost to follow up. The overall follow up ranged from 25 days to 14 years and nine months. The median survival period was 18 months.

A total of 80% of cases showed allelic imbalance/loss of heterozygosity for at least one microsatellite marker (fig 1). At the DSS82 locus, allelic imbalance/loss of heterozygosity was seen in five of 55 informative cases (table 2), at the DSS210 locus in seven of 54 cases, at the DSS299 locus in 11 of 41 cases, and at the DSS346 locus in 18 of 74 cases. In contrast, no microsatellite instability of at least one marker was seen in 16% of cases.

There appeared to be an association between sex and the microsatellite changes, although this association was not significant. For marker DSS299, a greater proportion of allelic imbalance/loss of heterozygosity was seen in male patients, whereas microsatellite instability was seen more frequently among female patients (p = 0.061). For marker DSS82, girls had a greater propensity for both allelic imbalance/loss of heterozygosity and microsatellite instability (p = 0.078; table 3).

For marker DSS82, allelic imbalance/loss of heterozygosity was observed in all stages, whereas the eight cases with microsatellite instability were all stage 4 tumours (p = 0.011; table 4). This marker also had a significant association with patient outcome (p = 0.027). All eight patients with microsatellite instability died (table 5).

No significant associations were found when preoperative chemotherapy (table 6), age at diagnosis, and histological classification were independently correlated with the results of microsatellite analysis. In addition, Kaplan–Meier survival plots showed no significant differences in survival between those patients with tumours that harboured APC microsatellite mutations and those that did not.

We found that β catenin was expressed in 93% of cases, and 35% of these cases showed membranous β catenin staining only (fig 2), 52% had both a membranous and cytoplasmic distribution pattern, and 6% displayed cytoplasmic staining only (table 7).

No significant relations were found when preoperative chemotherapy (p = 0.545), age at diagnosis, and histological classification were independently correlated with sex, clinicopathological stage, histological classification, and patient outcome. They were assessed by cross tabulation and the significance was determined by the χ² test. Differences were considered significant at p < 0.05. Survival curves were estimated by means of the Kaplan–Meier method, and differences in survival were evaluated with the log rank test. For the molecular study, only informative cases were analysed.
expression and APC microsatellite mutations.

Inactivation of gatekeeper genes is a rate limiting step for the initiation of a tumour, and both copies must be altered for tumour development. Mutations in other genes may not lead to sustainable growth perturbation in the presence of normal gene encoding the transforming growth factor \(\beta\) II receptor). Loss or mutation of APC is an early event in the tumorigenic process known to trigger the "adenoma–carcinoma" sequence of colorectal tumours. Mutations or allelic deletions of the APC gene are present in nearly 80% of sporadic colorectal carcinomas. In addition to colorectal cancers, somatic mutations of APC have been described in gastric cancer, hepatoblastoma, pilomatrixcoma, synovial sarcoma, medulloblastoma, and oesophageal cancer. Biallelic loss of APC has also been demonstrated in hepatoblastoma and hepatocellular adenoma. In our present study, allelic imbalance/loss of heterozygosity and microsatellite instability, followed by markers D5S299, D5S82, and D5S210. The informativity of the APC markers in our cohort was lower than that published by the Genome Database. Previous microsatellite studies in similar populations have also found very low informativity rates.

Marker D5S346 was the most informative marker, followed by markers D5S82, D5S210, and D5S299. In addition, marker D5S346 showed the highest number of aberrations (allelic imbalance/loss of heterozygosity and microsatellite instability), followed by markers D5S299, D5S82, and D5S210. The informativity of the APC markers in our cohort was lower than that published by the Genome Database. Previous microsatellite studies in similar populations have also found very low informativity rates.

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"Microsatellite instability appears to be a late event in nephroblastomas because all eight patients with microsatellite instability at marker D5S82 had stage 4 tumours, and these eight patients also had a poor outcome"
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The APC gene may not be the target of loss of heterozygosity in nephroblastoma because the frequency of APC loss was fairly low. Deletions involving multiple chromosomal loci have been reported in several human cancers. Genes on chromosome 5q other than APC include the IRF-1 (interferon regulatory factor 1) gene, the cytokine genes interleukin 3 (IL-3), IL-4, IL-5, and GM-CSF (granulocyte-macrophage colony stimulating factor), and the mitotic inducer CDC25C gene. Loss of 5q distinct from the APC locus has been reported in carcinomas of the lung, liver, and ovary, and in acute myelogenous leukaemia and myelodysplastic syndrome. Another tumour suppressor gene from the 5q21 region, MCC (mutated in colorectal cancer), has also been implicated in the pathogenesis of sporadic colorectal, lung, and oesophageal cancers. The mismatch repair gene hMSH3 and tumour suppressor gene del-27 are also located on the long arm of chromosome 5.

The immunoexpression of \(\beta\) catenin has been investigated in a wide variety of human malignancies where loss and aberrant staining patterns have been associated with tumour dedifferentiation and a poor outcome. In our study \(\beta\) catenin was expressed in 93% of cases. Consistent with other studies, there appears to be no appreciable loss of \(\beta\) catenin in nephroblastoma. \(\beta\) Catenin had a membranous localisation in 88% of cases, and was expressed not only in the epithelium but also in the mesenchyme and blastema. Nuclear relocalisation of \(\beta\) catenin has been associated with a poor outcome in tumours such as hepatoblastoma and synovial sarcoma. Nuclear relocalisation of \(\beta\) catenin has been associated with a poor outcome in tumours such as hepatoblastoma and synovial sarcoma. Previous immunohistochemical studies on nephroblastomas have also noted cytoplasmic and nuclear \(\beta\) catenin staining, but this finding was not associated with clinicopathological parameters. Although cytoplasmic \(\beta\) catenin staining was seen, according to Jass and colleagues this is not classed as abnormal \(\beta\) catenin immunolocalisation, and none of the cases achieved a score of more than 3. However, patients with a score of 3 had a shorter survival, although this finding may be stage dependent, and not necessarily related to the pronounced

<table>
<thead>
<tr>
<th>Score</th>
<th>No of cases</th>
<th>Type of staining</th>
<th>No of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>35</td>
<td>Membranous only</td>
<td>35</td>
</tr>
<tr>
<td>1</td>
<td>39</td>
<td>Loss of staining (both membranous and cytoplasmic)</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Membranous staining with slight cytoplasmic staining</td>
<td>32</td>
</tr>
<tr>
<td>2</td>
<td>22</td>
<td>Loss of membranous staining with slight cytoplasmic staining</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Membranous staining with pronounced cytoplasmic staining</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>Loss of membranous staining with pronounced cytoplasmic staining</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td></td>
<td>100</td>
</tr>
</tbody>
</table>
cytoplasmic staining and associated loss of membranous staining.

The cytoplasmic redistribution of β-catenin within a tumour may be a result of either Wnt signalling, inactivation of APC, or direct mutation of β-catenin itself. However, the role of the APC gene has been somewhat controversial, as tumours harboring a mutation in the APC gene may still exist. Interactions of β-catenin with several molecular partners have been shown to be important for cell signalling and cell-cell adhesion. Molecules such as E-cadherin, α-catenin, the epidermal growth factor receptor, and fascin, among many others, interact with β-catenin. The functional consequences of some of these interactions are well established, whereas the importance of others is still unclear. Changes in β-catenin concentrations will probably also impact on the function of these molecules and vice versa. We found no significant associations between β-catenin immunoreactivity and APC mutations. Although β-catenin retained its expression and relatively few APC mutations were seen, abnormalities in other members of the APC–β-catenin signalling pathway may still exist.

The administration of chemotherapy or radiotherapy may result in diverse morphological changes in tumours. These include maturation and cytodifferentiation in primitive embryonal tumours of childhood, including nephroblastomas. Although the exact mechanism for this phenomenon has not been identified, underlying genetic changes appear to be a prerequisite for these phenotypic alterations. In our study, most patients were treated with preoperative chemotherapy and this may have induced genetic alterations. Although no significant difference was noted when the results of APC analysis and β-catenin expression were correlated with the administration of preoperative chemotherapy, we are uncertain about the existence or nature of genetic changes attributable to preoperative chemotherapy in our patients because no prechemotherapy tumour tissue was available for analysis.

In conclusion, our study has shown that allelic imbalance/loss of heterozygosity of the APC locus is a more frequent aberration than microsatellite instability in this cohort of nephroblastomas. Although there was a significant correlation between some of the individual markers and the clinicopathological markers, the overall results do not support a prognostic role for the APC gene in nephroblastoma. In addition, there are no significant correlations between the expression of β-catenin and APC microsatellite mutations.

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