Elevated Expression of Wnt Antagonists Is a Common Event in Hepatoblastomas

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Abstract

Hepatoblastomas are the most frequent malignant liver tumors of childhood. A high frequency of activating β-catenin mutations in hepatoblastomas indicates that the Wnt signaling pathway plays an important role in the development of this embryonic neoplasm. Stabilization of β-catenin leads to an increased formation of nuclear β-catenin-T-cell factor complexes and altered expression of Wnt-inducible target genes. In this study, we analyzed the mRNA expression levels of nine Wnt genes, including c-JUN, c-MYC, CYCLIN D1, FRA-1, NKD-1, ITF-2, MMP-7, uPAR, and β-TRCP, by competitive reverse transcription-PCR. We analyzed 23 hepatoblastoma biopsies for which matching liver tissue was available, 5 hepatoblastoma cell lines, and 3 human fetal liver samples. β-TRCP and NKD-1 were highly expressed in all hepatoblastoma samples, independent of the β-catenin mutational status, in comparison with their nontumorous counterparts. β-TRCP mRNA overexpression was associated with accumulation of intracytoplasmic and nuclear β-TrCP protein. In human liver tumor cells without β-catenin mutations, Nkd-1 inhibited the Wnt-3a-activated Tcf-responsive-luciferase reporter activity, whereas Nkd-1 in hepatoblastomas with β-catenin mutations had no antagonistic effect. Our data emphasize the inhibitory effect of β-TrCP and Nkd-1 on the Wnt signaling pathway in a manner analogous to Conductin (AXIN2) and Dkk-1, inhibitors shown previously to be up-regulated in hepatoblastomas. Our findings indicate that overexpression of the Wnt antagonists Nkd-1 and β-TrCP reveals an activation of the Wnt signaling pathway as a common event in hepatoblastomas. We propose that Nkd-1 and β-TrCP may be used as possible diagnostic markers for the activated Wnt signaling pathway in hepatoblastomas.

Wnts are secreted glycoproteins that play a major role in the regulation of cell proliferation and differentiation during development. Activation of the Wnt signaling pathway, which is a common event in the development of various human cancers (1), leads to stabilization and accumulation of β-catenin, one of the main components in this pathway (2, 3). Cytoplasmic β-catenin abundance is controlled by the negative regulators AXIN1 and Conductin (AXIN2), which functionally interact with APC and GSK3β in a multiprotein complex (4, 5). This multiprotein complex promotes NH2-terminal phospho-
Most studies in which Wnt target genes have been identified and mRNA expression was analyzed were done on colorectal tumors and ovarian endometrioid adenocarcinomas. In these human cancers, the expression of presumptive target genes was documented and correlated with the mutational status of $\beta$-catenin and the nuclear accumulation of $\beta$-catenin protein. For instance, in samples from 70 patients with colorectal cancers, a correlation between deregulated $\beta$-catenin and cyclin D1 expression was shown by immunohistochemical analyses (22). Furthermore, a correlation between $\beta$-catenin mutations and increased expression of MMP-7 and uPAR was reported in colorectal tumors (23, 24). Zhai et al. analyzed the expression of six putative Wnt target genes in a panel of 44 primary ovarian endometrioid adenocarcinomas. Five genes revealed altered expression in cases with $\beta$-catenin mutations in contrast to the subset of tumors without mutations in the $\beta$-catenin gene (25). In hepatoblastomas, a correlation between $\beta$-catenin mutations and CYCLIN D1 expression but not with c-MYC overexpression was found (26).

In contrast, very few studies have been carried out with other tumor types as hepatoblastomas. Moreover, the relationship between gene expression in tumorous tissue and the respective normal tissue has not been documented in detail.

To find out whether the expression of known Wnt-inducible genes is altered in hepatoblastomas, in which Wnt signaling is activated, we investigated the expression of nine candidate genes in a panel of 23 hepatoblastoma cases for which matching normal liver tissue was available.

**Materials and Methods**

**Patients, tumors, and cell lines.** A total of 23 hepatoblastoma biopsies and 5 hepatoblastoma cell lines from sporadic hepatoblastomas were analyzed. Furthermore, we analyzed the cell line HepG2 derived from a childhood hepatocellular carcinoma (27). The age of patients with hepatoblastomas varied between 4 months and 12 years (Table 1). As control tissues, matching normal liver of each patient and three fetal livers (gestational age, 13.5-18 weeks) were available. Informed consent was given by the parents of all patients for the hepatectomy of each sample. As control tissues, matching normal liver tissue was available.

**RNA extraction and cDNA synthesis.** Total cellular RNA was extracted by lysis in guanidinium isocyanate and ultracentrifugation through a cesium chloride cushion or by Trizol reagent according to the protocol of the supplier (Life Technologies). To remove any contaminating genomic DNA, RNA was digested with 10 units RNase-free DNase I (Promega, Madison, WI) and 40 units RNasin (Promega) in a volume of 30 μl before reverse transcription. The RNAs were reverse transcribed using the SuperScript II Preamplication System (Life Technologies) with random hexamers as primers. Absence of contaminating genomic DNA was confirmed by amplification of a PCR fragment of the CDK4 gene with primers spanning an intronic sequence. All individual tissue samples selected for RNA extraction were preexamined by frozen section histology to document the histopathologic appearance of the specimen and to exclude contaminating necrotic or normal tissue. Only fragments with a tumor cell content of at least 80% were used for RNA extraction.

**$\beta$-catenin mutation analysis in hepatoblastoma samples.** All hepatoblastoma biopsies and all hepatoblastoma cell lines were examined for point mutations and deletions in exon 3 of the $\beta$-catenin gene using PCR/reverse transcription-PCR (RT-PCR) and single-strand conformational polymorphism analysis. The PCR conditions and primer sequences for single-strand conformational polymorphism analysis and the conditions for the detection of deletions are described elsewhere (13).

**mRNA expression of Wnt-inducible genes in hepatoblastomas by semiquantitative reverse transcription-PCR.** To measure gene expression levels, we first created exogenous RNA standards with internal deletions of $\approx 20$ bp for all nine Wnt-inducible genes and the housekeeping gene $\beta$-microglobulin by in vitro mutagenesis and in vitro transcription as described elsewhere (30, 31). Resulting cDNA templates (500 ng) were used for in vitro transcription (T7-polymerase kit, Fermentas, St. Leon-Rot, Germany). The resulting competitor RNA was DNase digested as described above and purified (RNaseasy Quickspin columns, Qiagen, Hilden, Germany) and the concentration was determined photometrically. To ensure coverage of the equimolar range of the competitors and the corresponding RNA transcripts in the final assay, a titration experiment was done. Total RNA (250 ng) of a pool from tumor and normal liver tissues was reverse transcribed together with defined amounts of exogenous standards (range, $5 \times 10^{-10} - 5 \times 10^{-6}$ mg). Reverse transcription was done as described above. The primers used for RT-PCR, the PCR conditions, and the fragment sizes of all analyzed Wnt genes and the housekeeping gene $\beta$-microglobulin are listed in Table 2. The reverse primer of each gene was fluorescently labeled with dye at the 5‘ position (FAM, JOE, MWG-Biotech, Ebersberg, Germany). Forward and reverse primers of each amplicon were chosen to span intronic sequences to exclude signals from contaminating genomic DNA. The resulting PCR products were separated on 4.5% denaturing acrylamide gels on a DNA sequencer (ABI377). The results were analyzed with the Genescan software (ABI, Darmstadt, Germany). Optimal titration was defined as the point of equal signal intensity of exogenous competitor and target transcript.

After determination of the optimal titration point of each exogenous standard found in the titration experiment, total RNA (250 ng) from each tumor and normal liver tissue were reverse transcribed together with the respective amount of each exogenous standard using the same procedure as described above. The objective was to ensure optimal coverage of even higher differences of expression level. The same primers and PCR conditions were used as described for the titration experiment. All PCRs were done thrice to increase the validity. The ratio of target PCR product intensity and standard intensity was used to determine the levels of gene expression. RNA expression levels were calculated by the following algorithm: $\text{TARGET}_{\text{sample}}/\text{TARGET}_{\text{standard}} / [\beta$-microglobulin]$_{\text{sample}}/[$β-microglobulin]$_{\text{standard}}$. The optimal titration for the genes in most of the samples was found for the following competitor amounts: 50 pg for $\beta$-microglobulin; 500 fg for c-MYC, FRA-1, ITF-2, c-JUN, MMP-7, human naked cuticle (NKD-1), and uPAR; and 100 fg for CYCLIN D1 and $\beta$-TRCP.

**Luciferase reporter assay.** HepT1, HUH6, and HUH7 cells were grown in 12-well plates in a volume of 1 ml. To avoid any effects from external factors, such as insulin, hepatoblastoma/hepatocellular carcinoma cell lines were washed with PBS and then transferred from their usual culture conditions to serum-free Neurobasal medium (Invitrogen, Karlsruhe, Germany) before transfection at an initial confluence of 50% to 60%. Cells were cotransfected with plasmids by lipofection (FuGene6, Roche, Mannheim, Germany). The following expression constructs were used: TOP-FACING (0.5 μg, containing three Tcf consensus-binding sites upstream of firefly luciferase cDNA) or FOP-FACING (0.5 μg, a plasmid with mutated Tcf-binding sites) reporters (Upstate, Hamburg, Germany), pcDNA-mNckD-1 (0.5 μg) expression construct, and NH2-terminally deleted $\beta$-catenin expression plasmid (0.5 μg; a kind gift from Dr. Walter Birchmeier, Max-Delbrück-Center for Molecular Medicine, Berlin, Germany). The Renilla SV40 construct (0.01 μg) was used as an internal control for transfection efficiency. The overall amount of DNA in transfection
mixtures was kept constant by addition of pcDNA3.1 (0.5 μg). Twenty-four hours after transfection, individual assays were costimulated with Wnt-3a (100 ng, R&D Systems, Wiesbaden, Germany) for another 24 hours. Individual assays were done in triplicates, and the luciferase/Renilla activity was measured using Dual-Luciferase Reporter Assay System (Promega) 48 hours following the transient transfection.

**Immunohistochemistry.** To determine whether the levels of transcripts of Wnt-regulated genes are associated with the nuclear accumulation of β-catenin protein, we did immunohistochemical staining of hepatoblastoma tissues. Sections of paraffin-embedded hepatoblastoma biopsies were available in 19 cases. The sections were stained with the monoclonal anti-β-catenin antibody clone 14 (IgG1, 0.25 μg/mL in PBS, 0.1% bovine serum albumin, Transduction Laboratories, Lexington, KY).

<table>
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<th>Tumor sample</th>
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<th>Specimen</th>
<th>Chemotherapy</th>
<th>Histology</th>
<th>β-Catenin mutation</th>
<th>Nuclear β-catenin accumulation</th>
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<td>Cell line of D166</td>
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Abbreviations: ND, no data; NA, not analyzed.

*In years.
Binding of the primary antibody was detected by avidin-biotin complex method (DAKO, Copenhagen, Denmark) and visualized by diamino benzidine tetrahydrochloride. The exact protocol for the β-catenin immunostaining has been described in a previous work (13).

To determine the patterns of subcellular localization of β-TrCP, sections were incubated with an antibody against β-TrCP (in PBS, 2 μg/mL). β-TrCP was detected using avidin-biotin complex method in microwaved tissue sections as described (13). Normal liver parenchyma adjacent to the tumor was available as an internal positive control in five cases.

**Statistical analysis.** After quantification of the signals by semiquantitative RT-PCR and normalization of the values to β-2-microglobulin levels, the expression of each Wnt-inducible gene in hepatoblastoma without Wnt tumor/liver tissue) of each sample was compared with the expression in the corresponding normal liver tissue. Student’s t test was used to determine the significance of difference in the expression levels (ratio hepatoblastoma/liver tissue) of each Wnt gene in hepatoblastomas with and without β-catenin mutations.

**Results**

**β-catenin mutations in hepatoblastoma samples.** All hepatoblastoma biopsies and cell lines used for expression analysis were examined for β-catenin exon 3 mutations by single-strand conformational polymorphism analysis. For the detection of larger deletions, we analyzed a region spanning exon 2 to exon 4 of the β-catenin gene by PCR/RT-PCR. Five of 23 hepatoblastoma cases harbored somatic point mutations (Table 1). Large deletions which lead to exon 3 deletion were identified in eight hepatoblastoma cases and in two of five hepatoblastoma tumor cell lines (Table 1).

**mRNA expression analysis.** mRNA expression of nine candidate Wnt-inducible genes in 23 hepatoblastoma biopsies and their corresponding liver tissues are shown in Table 3. In hepatoblastomas, expression levels were calculated as ratios of the expression in the tumor normalized to the expression in the respective liver tissue. An expression level of >200% was scored as overexpressed, 50% to 200% as no difference, and <50% as repressed.

**CYCLIN D1, c-MYC, MMP-7, BRAF-1, uPAR, c-JUN, and IFT-2 showed no statistically significant differences in the expression levels of the tumors compared with the corresponding liver tissues in our set of 23 hepatoblastomas (t test). Furthermore, t tests did not show a significant correlation of the β-catenin mutational status and the expression of the CYCLIN D1, c-MYC, MMP-7, BRAF-1, uPAR, c-JUN, and IFT-2 genes (Table 3).** There was no statistical difference (t test) in the expression of the cell lines compared with fetal and adult liver tissue (data not shown).

For instance, CYCLIN D1 transcripts were expressed at higher levels in tumors from 12 of the 23 patients compared with their normal corresponding liver tissue. The increase in the level of mRNA transcript (as ratio tumor/liver) ranged from 3.23 to 137.49 (mean, 23.96). Three cases showed no difference in the expression and eight cases showed reduced levels in the tumor compared with liver ranging from 0.03 to 0.34 (Table 3). There was no statistically significant correlation between the expression levels of CYCLIN D1 and the presence of activating β-catenin mutations (P = 0.41, t test).

**mRNA expression of NKD-1.** All hepatoblastomas showed a significant up-regulation of NKD-1 mRNA compared with adjacent normal liver tissues. The tumor to normal tissue mRNA ratio ranged from 6.63 to 517.73 (mean, 179.32; Table 3; Fig. 1A). Neither a correlation between the expression levels of NKD-1 and the presence of activating β-catenin mutations (P = 0.95, t test) nor a correlation between NKD-1 expression and tumor histology could be found (Fig. 2). All cell lines revealed increased NKD-1 expression (range, 3.64-107.96;
Table 3. Results of the mRNA expression analysis of the Wnt genes in 23 hepatoblastomas

<table>
<thead>
<tr>
<th>Gene</th>
<th>β-catenta-1 mutations (13 hepatoblastoma cases)</th>
<th>No β-catenta-1 mutations (10 hepatoblastoma cases)</th>
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<tbody>
<tr>
<td></td>
<td>Ratio hepatoblastoma/liver &gt;200%</td>
<td>Ratio hepatoblastoma/liver 50-200%</td>
</tr>
<tr>
<td>CYCLIN D1</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>FRA-1</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>ITF-2</td>
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<td>9</td>
</tr>
<tr>
<td>c-jUN</td>
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<td>6</td>
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<td>MMP-7</td>
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<td>c-MYC</td>
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<td>5</td>
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<td>β-TRCP</td>
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<td>0</td>
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<tr>
<td>uPAR</td>
<td>8</td>
<td>4</td>
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NOTE: The mRNA levels were determined by competitive RT-PCR. The factors are calculated as ratios of mRNA expression in tumor versus expression in the adjacent normal liver tissue. Expression level >200% was defined as overexpressed, 50% to 200% as no difference, and <50% as repressed. The significance of the expression in cases with and without β-catenta-1 mutations was determined with the t test.

**Fig. 1.** Results of the mRNA expression analysis of NKD-1 (A) and β-TRCP (C) in 23 hepatoblastomas. The expression levels were determined by competitive RT-PCR. Ratio of mRNA expression in tumor versus mRNA expression of the adjacent normal tissue. Student’s t test was used to determine the significance of differences in NKD-1 and β-TRCP expression in hepatoblastomas with and without β-catenta-1 mutations. NKD-1 (B) and β-TRCP (D) mRNA levels in human hepatoblastoma cell lines, a hepatocellular carcinoma cell line (HepG2), and fetal liver tissue. mRNA expression was normalized to β2-microglobulin. Expression levels were compared with the mean values of the liver tissue.
mean, 39.87 (ratio NKD-1/β2-microglobulin) compared with the average NKD-1 expression level in the normal liver tissue (mean, 0.11; Fig. 1B). In addition, the cell line HepG2, which was derived from a pediatric hepatocellular carcinoma biopsy, also revealed high NKD-1 mRNA levels. There was no difference in NKD-1 mRNA levels between fetal and adult liver tissues.

**mRNA expression of β-TRCP.** Determination of the β-TRCP mRNA levels by competitive RT-PCR revealed that all hepatoblastomas displayed transcript levels higher than the corresponding normal liver tissue. mRNA expression levels ranged from 1.36 to 48.58 (mean, 9.33; Table 3; Fig. 1C). Expression differences for β-TRCP between tumors with β-catenin mutations and tumors without β-catenin mutations were not apparent (P = 0.40, t test). The five hepatoblastoma cell lines and the hepatocellular carcinoma cell line HepG2 showed an increased expression of β-TRCP (range, 1.36-75.59; mean, 24.55) compared with the average expression level in the normal liver tissue (mean, 0.17; Fig. 1D). There was no significant difference in β-TRCP expression between fetal and adult liver tissues.

**β-catenin immunostaining.** Immunostaining with antibodies against β-catenin showed a nuclear accumulation of β-catenin protein in all of the 19 analyzed cases (Fig. 2A and C). Hepatoblastomas were next evaluated for expression and localization of β-TrCP protein by immunohistochemistry. Consistent with the mRNA data, the normal liver parenchyma showed weak cytoplasmic staining of β-TrCP (Fig. 3B). In contrast, hepatoblastomas showed a constitutively cytoplasmic distribution of β-TrCP (Fig. 3A). In agreement with other studies, nuclear accumulation of β-TrCP protein was also observed in cases with nuclear accumulation of β-catenin protein (32).

**Nkd-1 antagonizes Wnt signaling in liver tumor cells.** The role of Nkd-1 in the Wnt signaling pathway in human hepatoblastoma and hepatocellular carcinoma cell lines was tested by using a Wnt-3a ligand-responsive TOP-FLASH/GFPLuciferase reporter assay (Fig. 3D). The hepatoblastoma cell lines, but not the hepatocellular carcinoma cell line HepG2, showed a significant decrease in luciferase activity upon treatment with Wnt-3a (Fig. 3D). These results suggest that Nkd-1 may act as an endogenous inhibitor of Wnt signaling in liver tumor cells.
FOP-FLASH luciferase reporter assay. First, cell line HUH7, derived from a human hepatocellular carcinoma and known to exhibit low levels of β-catenin and harbor no known mutations in any of the genes encoding for components of the multiheterogeneous complex, was stimulated with Wnt-3a. This resulted in a 2.5-fold increase in the TOP-FLASH luciferase activity (Fig. 4A). Activation of the TOP-FLASH reporter by Wnt-3a was completely inhibited by coexpression of Nkd-1. Expression of Nkd-1 in the absence of Wnt-3a down-regulates the TOP-FLASH luciferase activity (factor, 0.66; Fig. 4A). Moreover, Nkd-1 failed to inhibit the gene response by overexpression of NH2-terminally deleted β-catenin.

No inhibitory effect of Nkd-1 in hepatoblastoma cell lines with β-catenin mutations. Next, we transfected the hepatoblastoma cell lines HepT1 and HUH6. Both cell lines carry activating mutations of the β-catenin gene, which was reflected in a marked TOP-FLASH luciferase activity. Cotransfection with Nkd-1 had no effect on the activity of the luciferase reporter in any of the cell lines (Fig. 4B). These data suggest that Nkd-1 is not able to antagonize the Wnt signal in tumors in which the Wnt signaling pathway is activated by mutations in β-catenin.

Discussion

Several components of the Wnt signaling pathway are altered in a variety of human cancers. Mutational defects result in nuclear accumulation of β-catenin protein and increased expression of specific Wnt target genes that play key roles in proliferation and survival of cancer cells. In particular, the expression of Wnt target genes has been well characterized in colon cancers and ovarian endometrioid adenocarcinomas. Because it is known that the Wnt signaling pathway is involved in the pathogenesis of sporadic hepatoblastomas by oncogenic activation of the β-catenin protein by amino acid substitutions and interstitial deletions in high frequency (10, 11, 13), we first investigated whether the expression of known Wnt-inducible genes is dependent on the mutational status of...
β-catenin. Expression analysis was done in 23 hepatoblastomas and their matching normal liver tissue as well as in 3 fetal liver samples and 6 cell lines.

We show here that mRNA transcripts of all nine analyzed Wnt genes were detectable in all samples examined. However, in contrast to colon carcinomas and ovarian endometrioid adenocarcinomas, the expression of c-JUN, c-MYC, FRA-1, ITF-2, MMP-7, uPAR, and CYCLIN D1 revealed no statistical significant differences between hepatoblastomas with and without β-catenin mutations (Table 3). Moreover, in each group (with/without β-catenin mutations) of our hepatoblastoma series, we compared gene expression of the tumor with normal liver tissue. No statistically significant correlation was observed (Table 3; Fig. 1A and C).

In particular, CYCLIN D1 was one of the first genes to be implicated as a Wnt-regulated target gene in hepatoblastomas (26). Our competitive RT-PCR assay revealed increased expression of CYCLIN D1 in 12 hepatoblastomas and a reduced expression in 8 of the 23 cases compared with corresponding liver tissue, whereas 3 cases showed no difference (Table 3). A significant correlation between the expression levels of CYCLIN D1 mRNA and the presence or absence of activating β-catenin mutations in hepatoblastomas could not be shown. Previous studies of the cyclin D1 expression in hepatoblastomas have yielded different results. Kim et al. (33) showed by immunohistochemistry in 17 paraffin-embedded hepatoblastoma tissues that 11 cases showed overexpression of cyclin D1, whereas Iolascon et al. (34) showed that CYCLIN D1 mRNA expression is reduced in hepatoblastomas compared with normal liver tissue.

Overall, the finding that CYCLIN D1 is heterogeneously expressed in hepatoblastomas is not a surprising result, as CYCLIN D1 expression is likely to be regulated by multiple different signaling pathways. For that matter, the above-listed genes were described as Wnt targets in different cancers. However, our results show a lack of association between an activated Wnt signaling pathway and expression of c-JUN, c-MYC, FRA-1, ITF-2, MMP-7, uPAR, and CYCLIN D1 in hepatoblastomas. These results are in line with the findings of other groups. For example, although a high degree of correlation between nuclear β-catenin accumulation and overexpression of c-myc protein was seen in colon tumors (35), similar studies in hepatoblastomas and breast cancers found no such correlation (26, 36). A possible explanation for the absence of a strict correlation between β-catenin mutations and gene expression is that β-catenin-binding proteins, such as Chibby or ICAT, might modulate β-catenin function and its ability to activate T-cell factor–regulated transcription (37, 38). A further explanation for the striking variation in the expression of the above-described Wnt target genes is that expression of these genes is controlled by other signaling pathways.

Interestingly, the Wnt-inducible genes β-TRCP and NKD-1 were found to be overexpressed in all analyzed hepatoblastoma samples in comparison with the respective tumor normal liver tissue. A comparison between cases with and without oncogenic β-catenin mutations revealed no significant difference in the expression level (Table 3; Fig. 1A and C). Moreover, our analysis revealed a higher expression of β-TRCP and NKD-1 mRNA in all hepatoblastoma and hepatocellular carcinoma cell lines compared with normal liver tissue (Table 3; Fig. 1B and D).

These findings prompted us to analyze the status of the β-catenin protein in the cases used for expression analysis. Of the 23 hepatoblastoma biopsies, paraffin-embedded material was available in 19 cases. All 19 hepatoblastomas showed a nuclear accumulation of β-catenin protein, support of activation of the Wnt signaling pathway regardless to the presence of β-catenin mutations.

It is important to note that β-TrCP and Nkd-1 encode for known inhibitors of the Wnt signaling pathway. β-TrCP is a component of the ubiquitin ligase complex targeting β-catenin for proteasomal degradation and is thus a negative regulator of Wnt signaling. Activation of the Wnt signaling pathway by Wnt-1 or overexpression of a stabilized β-catenin mutant in human embryo kidney 293T cells resulted in an increase of β-TRCP mRNA (7), indicating an induction of β-TrCP by the Wnt signaling pathway.

Apart from the induction of β-TRCP mRNA by the activated Wnt signaling pathway in hepatoblastomas, we found that in hepatoblastomas increased levels of β-TrCP protein were associated with nuclear accumulation of β-catenin (Fig. 3A). Immunohistochemistry showed that β-TrCP protein was accumulated in both the cytoplasm and the nucleus of hepatoblastomas cells as in their respective normal liver cells, which only showed a weak β-TrCP expression (Fig. 3B). These results agree with the studies of Ougolkov et al. in which increased levels of β-TrCP protein were found in colorectal cancers compared with colorectal crypt epithelial cells (32).

The gene encoding NKD-1 has been identified as a further antagonist of the Wnt signaling pathway. Acting upstream of β-catenin, Nkd-1 targets dishevelled to antagonize Wnt signal transduction (39, 40), and a putative calcium-binding (EF hand) motif is required for the inhibitory function (41). Yan et al. (41) showed that Nkd-1 inhibits the Wnt-1 activated lymphoid enhancer factor-1 luciferase reporter in human embryo kidney 293 cells. Moreover, mRNA expression of NKD-1 was shown to be increased in colon cancer cell lines with oncogenic β-catenin mutations (41). Having found NKD-1 being overexpressed in comparison with the matching normal liver tissue in all hepatoblastoma samples analyzed (Table 3; Fig. 1), we wanted to know to which extent Nkd-1 has the possibility to antagonize Wnt signaling in liver tumor cells with and without β-catenin mutations.

The role of Nkd-1 in the Wnt pathway was first tested in the human liver tumor cell line HUH7 (no β-catenin mutation/no β-catenin accumulation) by using a Wnt-3a ligand-responsive luciferase reporter assay. Activation of the TOP-FLASH reporter by Wnt-3a was completely inhibited by coexpression of Nkd-1. Coexpression of Nkd-1 with mutated Δβ-catenin had no inhibitory effect on the activity of the reporter in HUH7 cells (Fig. 4A). The same luciferase reporter assays were done in the hepatoblastoma cell lines HepT1 and HUH6, which carry mutations in the β-catenin gene. Here, overexpression of Nkd-1 had no antagonistic effect on the activity of the reporter (Fig. 4B). These results are in line with observations of other groups who showed that Nkd-1 performs its inhibitory action upstream of β-catenin and thus is not capable of decreasing the Wnt signaling in the cells that have already accumulated β-catenin. It allows to assume that, most likely, alterations in the Wnt pathway downstream of Nkd-1 cause nuclear accumulation of β-catenin protein in hepatoblastomas.
without \(\beta\)-catenin mutations or that other signaling pathways affect Tcf transcription downstream of Nkd-1.

It is important to note that although \(\beta\)-TRCP and NKD-1 are overexpressed in all hepatoblastomas compared with the normal liver tissue, there is a variation in the up-regulation of these genes in hepatoblastomas. As above mentioned, a possible explanation for this finding is that signaling pathways other than Wnt could contribute to induction of \(\beta\)-TRCP and NKD-1 expression. For example, the phosphatidylinositol 3-kinase/Akt pathway modulates \(\beta\)-catenin/T-cell factor–mediated gene transcription through inactivation of GSK3\(\beta\) and nuclear accumulation of \(\beta\)-catenin in prostate cancer cells (42). In addition, the above-described \(\beta\)-catenin-binding proteins Chibby and ICAT could contribute to the variation in the levels of \(\beta\)-TRCP and NKD-1 mRNA expression. Nevertheless, up-regulation of \(\beta\)-TRCP and NKD-1 mRNA transcripts are detectable in all analyzed hepatoblastoma biopsies in contrast to the other Wnt target genes in which no significant overexpression could be found. Interestingly \(\beta\)-TrCP and Nkd-1 encode for known antagonists of the Wnt pathway.

Very recently, two other antagonists of the Wnt signaling pathway, Dkk-1 and Conductin (AXIN2), have been identified to be significantly overexpressed in hepatoblastomas. DKK-1, a direct target gene of Wnt signaling (43, 44), antagonizes Wnt by blocking the LRP6-Frizzled complex (45, 46). We identified DKK-1 as a differentially expressed gene in hepatoblastomas using the subtractive suppression hybridization method. Therein, we showed that >80% of the analyzed hepatoblastomas showed overexpression of the DKK-1 gene (47), suggesting the existence of a novel but apparently nonfunctional Wnt feedback loop in hepatoblastomas (Fig. 5).

The findings of Lustig et al. (17) indicate that the Wnt antagonist Conductin (AXIN2) as a direct target gene of the Wnt signaling pathway constitute an essential compound of a negative feedback mechanism of Wnt signaling. We examined the AXIN2 expression levels in our set of hepatoblastoma biopsies and in the corresponding liver samples using competitive RT-PCR and Western blot. Interestingly, hepatoblastomas showed AXIN2 overexpression in comparison with the liver tissue (15).

These findings support the view that increased expression of Wnt-inducible genes, such as AXIN2, DKK-1, NKD-1, and \(\beta\)-TRCP, which act as inhibitors of the Wnt pathway, is a common event in hepatoblastomas. This indicates that activation of the Wnt pathway is present in most hepatoblastoma tissues. The biological capability of these antagonists to inhibit Wnt signaling through a negative feedback
loop seems to be abrogated in hepatoblastomas, most likely because genetic alterations disrupt the central multi-protein complex that controls the stability of β-catenin (Fig. 5). Because the strong expression of Wnt antagonists is a common theme in hepatoblastomas, we propose that Axin2, Dkk-1, β-TrCP, and Nkd-1 may be used as biological and diagnostic markers for these embryonic liver malignancies.

References

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