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A novel tissue-based ß-catenin gene and immunohistochemical analysis to exclude familial adenomatous polyposis among children with hepatoblastoma tumors

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Abstract

Background: The Wnt/β-catenin pathway plays a central role in the pathogenesis of most hepatoblastomas (HBs), that is, up to 60–80% carry activating *CTNNB1* mutations. HBs can however also be the first manifestation of familial adenomatous polyposis (FAP). As this is a severe disease, it is important for the patient and related family members to firmly exclude FAP at an early stage. Current diagnosis largely depends on *APC* germline mutation detection on genomic DNA, which is associated with 10–20% false-negative results. Here, we establish and validate a tissue-based β-catenin gene and immunohistochemical analysis, which complements germline mutation screening to exclude the diagnosis of FAP among HB patients.

Methods: Tumor tissues of 18 HB patients, including three FAP cases were subjected to *CTNNB1* exon 3 mutational analysis and immunohistochemistry comparing staining patterns for total and exon 3 specific β-catenin antibodies.

Results: Our novel tissue-based method reliably identified all three FAP patients. Their tumors were characterized by a wild-type exon 3 sequence and a comparable nuclear staining for both antibodies. In contrast, the non-FAP tumors carried missense *CTNNB1* mutations combined with a clearly reduced staining for the exon 3 antibody, or complete loss of staining in case of lesions with exon 3 deletions.

Conclusion: We have successfully established and validated a novel ß-catenin gene and immunohistochemical diagnostic method, which, when combined with routine germline DNA testing, allows the exclusion of the diagnosis of FAP among HB patients.

KEYWORDS

APC, CTNNB1, familial adenomatous polyposis (FAP), genetic counseling, hepatoblastoma, Wnt/β-catenin signaling

1 | INTRODUCTION

Hepatoblastoma (HB) is the most common pediatric liver malignancy with an estimated incidence of 1 per 100,000 children, mainly affecting children during their first 3 years of life.[1,2] HBs are believed to originate from hepatic progenitor cells that acquire malignant transformation during embryogenesis. Histologically they show similarity to immature hepatocytes of the developing liver.[3,4] Most HBs are believed to be of sporadic nature, but about 10–20% have been associated with genetic defects such as

Abbreviations: BWS, Beckwith–Wiedemann syndrome; FAP, familial adenomatous polyposis; FFPE, formalin-fixed paraffin-embedded; HB, hepatoblastoma; HCA, hepatocellular adenoma; HCC, hepatocellular carcinoma; IHC, immunohistochemistry; LFS, Li–Fraumeni Syndrome; NF1, neurofibromatosis type 1; S/T, serine/threonine

Beckwith–Wiedemann syndrome (BWS) or familial adenomatous polyposis (FAP).[2,5,6]

The Wnt/β-catenin signaling pathway is recognized as having a central role in the pathogenesis of HBs.[4] The β-catenin protein is a key effector of the pathway, affecting cellular decisions such as stem cell maintenance and cell proliferation through modulating the expression of specific target genes. In normal cells, β-catenin is involved both in cell adhesion, when located at the cell membrane, and in transcriptional regulation, when present in the nucleus. In the absence of upstream Wnt signaling, β-catenin is phosphorylated at N-terminal serine/threonine (S/T) residues by a multiprotein complex consisting of the adenomatous polyposis coli (APC) tumor suppressor, scaffold proteins AXIN1, AXIN2, and AMER1, and the kinases GSK3 and CK1α.[7–11] Phosphorylated β-catenin is then ubiquitinated, leading to its proteasomal degradation. When cells are exposed to Wnt ligands, this β-catenin breakdown complex is temporarily inhibited, leading to the stabilization of β-catenin. As a result, it translocates into the nucleus and associates with members of the TCF/LEF family of transcription factors, thus regulating the expression of specific downstream Wnt/β-catenin target genes.

In several tumor types, this pathway is constitutively activated due to "loss of function" mutations of the *APC*, *AXIN1*, or *AXIN2* genes leading to inefficient β-catenin degradation and its intracellular stabilization. Other tumors carry oncogenic β-catenin (*CTNNB1*) mutations within exon 3 at the N-terminal phosphorylation residues, making the protein more resistant to proteolytic degradation. Important for our study, disease-causing mutations in the *APC* and *CTNNB1* genes have been shown to occur in a mutually exclusive manner in all tumor types studied so far.[12] These mutations lead to aberrant stabilization of β-catenin, which constitutively activates downstream Wnt/β-catenin target genes and triggers a genetic program resulting in tumor formation.

HBs are characterized by a high proportion (60–80%) of activating mutations within the *CTNNB1* gene, either by point mutation or deletions encompassing exon 3.[13–22] The deletions represent more than 60% of the somatic *CTNNB1* mutations and vary in size from small intra-exonic deletions to larger ones extending up to part of exon 2 or 4. All of them have in common that they result in in-frame deletions leading to mutant β-catenin that is resistant to degradation.

Somatic *APC* mutations have been rarely reported in HBs.[14,16,17,23] However, FAP patients carrying a germline *APC* mutation present with a 750–7500-fold increased risk of HB development in comparison to the general population.[2,24–27] FAP is a hereditary predisposition to develop hundreds to thousands of colorectal polyps ultimately leading to colorectal cancer. Untreated, the risk of colorectal cancer in FAP patients approaches 100%. The average age when colorectal polyps are detected is about 15 years.[28] Colonoscopy and surgery in adolescence prevent colorectal cancer formation in FAP. When FAP is diagnosed in time, health benefits and increased life expectancy can be achieved. Importantly, in children HB can be the first manifestation of FAP within a family, especially given the high proportion (10–25%) of de novo germline mutations occurring in the *APC* gene.[29–31] This is of importance for the management of the child and its possible future offspring, but it might also have implications for yet asymptomatic parents and siblings. For this reason, it has been advocated that all HB patients with unknown family history should be referred for *APC* germline mutation detection,[6,32–34] using a combination of currently available methods, such as next-generation or Sanger sequencing, and multiplex ligation-dependent probe amplification (MLPA). These methods will uncover up to 80–90% of disease-causing *APC* mutations,[35,36] but inherent to any diagnostic method will lead to false-negative results. Examples include complex insertions and deletions, chromosomal translocations, or instances in which the patient is a low-mosaic carrier of the *APC* mutation.[37,38] Moreover, sequence analysis will identify genetic variants with uncertain disease-causing relevance. Given the severity of the FAP syndrome, a thorough exclusion of carrier status is highly desirable. Here, we establish and validate a tissue-based β-catenin gene and immunohistochemical analysis, which complements the germline mutation screening to exclude the diagnosis of FAP among HB patients. We also discuss options regarding how it can be implemented in current clinical practice.

2 | MATERIALS AND METHODS

2.1 | Patients

From the period 1995–2013, in total 23 pediatric liver tumors were available for analysis from the pathology archive of the Erasmus MC. Tissues were obtained from the initial operative procedure and embedded in paraffin after formalin fixation. The medical records and family history of patients were analyzed. Patient and tumor characteristics are provided in Table 1. All samples were revised by a GI-pathologist (MD) according to a recently proposed consensus classification for pediatric liver tumors.[39]

2.2 | Immunohistochemistry (IHC)

Immunohistochemical analyses for total β-catenin (1:200, clone-14, BD-Transduction Laboratories) and nonphosphorylated Ser33/37/Thr41 β-catenin (1:400, #8814, Cell Signaling Technology) were performed in an automated stainer (Benchmark-Ultra, Ventana Medical Systems, Tucson, AZ). Sections were deparaffinized and pretreated with standard cell conditioning 1 solution (CC1) at 100°C for 64 min, followed by incubation with the specified antibodies at 37°C for 60 min. The antibodies were visualized with the OptiView IHC DAB Detection Kit.

2.3 | Tumor DNA and RNA isolation and *CTNNB1* mutation analysis

Tumor DNA was extracted by microdissection from formalin-fixed paraffin-embedded (FFPE) tissue fragments using proteinase-K and 5% Chelex®-100 Chelating Resin (BioRAD, #1432832), as previously described.[40] Sequence analysis of CTNNB1 exon 3 was performed by bidirectional sequencing of PCR-amplified fragments using M13-tailed forward and reverse primers (Supplementary Material S1). To test for

TABLE 1 Patient and tumor characteristics

Sample	Gender	Age (years)	HB/HCC	Pathology	Known genetic predisposition
HB-4	F	5.5	HB	Epithelial (too few tumor cells for proper evaluation)	Familial adenomatous polyposis
HB-6	M	0, 6	HB	Epithelial mixed	
HB-7	M	2, 1	HB	Epithelial fetal	
HB-8	M	5, 9	HCC	Classic	
HB-11	F	1.2	HB	Epithelial mixed	
HB-13	F	0.5	HB	Epithelial mixed	
HB-14	M	3.5	HB	Epithelial fetal	Familial adenomatous polyposis
HB-15	F	9.7	HCC	Classic	Belonging to Li–Fraumeni family (TP53)
HB-16	F	15.8	HCC	Fibrolamellar	
HB-17	M	9.7	HB	Epithelial fetal with low mitotic activity	
HB-18	F	11.4	HCC	Fibrolamellar	
HB-19	F	1.7	HB	Epithelial fetal with low mitotic activity	Hypomethylation of LIT1 (no signs of BWS)
HB-20	M	1, 1	HB	Mixed epithelial-mesenchymal, no teratoid	See note[a]
HB-21	M	0.8	HB	Epithelial mixed	
HB-22	M	1.0	HB	Epithelial mixed	
HB-23	M	9.4	HB	Epithelial fetal	
HB-24	M	1.8	HB	Epithelial fetal, mitotically active	
HB-25	F	16.3	HCA		See note[a]
HB-27	F	7.3	HB	Epithelial pleomorphic	
HB-28	F	1.1	HB	Mixed epithelial-mesenchymal, teratoid	
HB-29	M	1.6	HB	Mixed epithelial-mesenchymal, teratoid	Familial adenomatous polyposis
HB-30	F	0.8	HB	Mixed epithelial-mesenchymal	
HB-32	F	1.1	HB	Mixed epithelial-mesenchymal, no teratoid	Neurofibromatosis type 1

HCA, hepatocellular adenoma; HB, hepatoblastoma; HCC, hepatocellular carcinoma.
[a]Suspicion of syndromic condition based on additional (congenital) abnormalities.

genomic deletions of CTNNB1 exon 3, two independent PCRs were performed using primers located within exon 2 and exon 4. Details of the PCR and sequencing reactions and RNA isolation are provided in Supplementary Material S1.

2.4 | Cell lines

Short Tandem Repeat-verified cell lines used in this study were cultured as previously described.[41] Preparation of paraffin-embedded cell line blocks is described in Supplementary Material S1.

2.5 | Generation of β-catenin variant expression vectors and transfection

Expression vectors for N-terminal FLAG-tagged β-catenin variants were generated using the pcDNA-5′UT-FLAG vector as basis (kindly provided by Dr. Veronique Lefebvre, Lerner Research Institute, Cleveland OH, USA). Wild-type, S33Y and exon 3 deleted variants of human *CTNNB1* were cloned using the Gibson assembly method (NEB). The G34V, S37F, T41A, and S45P variants were generated by Q5® site-directed mutagenesis (NEB), using the wild-type clone as basis. Primers are available upon request. All plasmids were fully sequence verified. Next, HEK-293 cells seeded in 6-well plates were transiently transfected with Fugene-HD (Promega) using 1 μg of plasmid DNA. After 2 days, cells were lysed in Laemmli sample buffer with 0.1 M DTT and heated for 5–10 min at 95°C.

2.6 | Western blot assay

The western blot assay and quantification for the FLAG-tag, total, and nonphosphorylated Ser33/37/Thr41 β-catenin were performed basically as previously described, using all antibodies at a 1:1000 dilution.[41]

3 | RESULTS

3.1 | Patient and tumor characteristics

In total, 23 pediatric liver tumors were included in the study (Table 1), of which 18 were HBs, four hepatocellular carcinomas (HCC), and one hepatocellular adenoma (HCA). In accordance with literature, most HB patients were diagnosed during their first 3 years of life. The youngest age at diagnosis was 0.5 years, whereas the oldest patient was 9.7 years old. HB patients HB-4, HB-14, and HB-29 had been diagnosed with FAP previously. The HCC detected in HB-15 was diagnosed in a child belonging to a Li–Fraumeni family (LFS), i.e. *TP53* germline mutation, whereas HB-32 was observed in a patient with neurofibromatosis type 1 (NF1). Hypomethylation of LIT1 was reported for patient HB-19, although no other signs of BWS were observed.

3.2 | IHC for total β-catenin

IHC for β-catenin showed nuclear accumulation of β-catenin in one HCC (HB-8), while in the remaining three HCCs and the HCA an exclusive membranous pattern was observed (Figure 1). All HBs showed evidence of nuclear staining, including the ones derived from the FAP patients. No nuclear β-catenin was detected in normal pre-existing hepatocytes.

3.3 | *CTNNB1* mutation analysis

Sequence analysis of exon 3 of *CTNNB1* succeeded in 22/23 tumors. In 8/18 HBs point mutations were detected within or in close proximity to the region encoding the S/T residues required for proteolytic breakdown of β-catenin (Figure 1). As expected, no mutation was detected in all three FAP-derived HBs and in the HCC and HCA samples without nuclear staining. Also no mutation was detected within the single HCC with nuclear accumulation of β-catenin.

HBs are however characterized by a high proportion of genomic deletions partially or completely encompassing exon 3,[13–21] most of which will be missed by the regular exon 3 sequence analysis. Therefore, we attempted two genomic PCRs on DNA isolated from the FFPE samples, using a common forward primer in exon 2 and two reverse primers, respectively at the 5′- and 3′-side of exon 4. The latter can detect the reported deletions extending up to the middle of exon 4.[13–21] Specific PCR products were detected only in three HB samples identifying two genomic deletions, both predicted to result in the loss of critical N-terminal phosphorylation sites (HB-30 and HB-32 in Figure 1). For the remainder of the samples the FFPE-isolated DNA quality was apparently of insufficient quality to generate PCR products with the deletion primers, meaning that no judgment can be made about a possible deletion. In summary, the DNA analysis identified 10 somatic *CTNNB1* mutations, all in HBs showing nuclear accumulation of β-catenin.

Sample	HB or HCC	genetic predisposition	Nucleotide change detected in genomic DNA	RNA alteration	Amino Acid alteration	C-ter β-cat IHC	S33/37/T41 β-cat IHC
HB-15	HCC	LFS	None			Membrane only	Membrane only
HB-16	HCC		None			Membrane only	Membrane only
HB-18	HCC		None			Membrane only	Membrane only
HB-25	HCA		None			Membrane only	Membrane only
HB-4	HB	FAP p.Q1062*	None			Nuclear	Nuclear
HB-14	HB	FAP p.R1114*	none			Nuclear	Nuclear
HB-29	HB	FAP p.Q1062*	None			Nuclear	Nuclear
HB-6	HB		c.97T>C		p.S33P	Nuclear	Nuclear
HB-17	HB		c.86C>T + c.94G>T		p.S29F + p.D32Y	Nuclear	Moderate Nuclear
HB-22	HB		c.94G>A		p.D32S	Nuclear	Nuclear
HB-7	HB		c.101G>T		p.G34V	Nuclear	Faintly nuclear
HB-13	HB		c.101G>T		p.G34V	Patchy nuclear	Faintly nuclear
HB-20	HB		c.101G>T		p.G34V	Nuclear	Faintly nuclear
HB-21	HB		c.101G>T		p.G34V	Nuclear	Faintly nuclear
HB-28	HB		c.121A>G		p.T41A	Nuclear	Patchy nuclear
HB-8	HCC		None	r.14_241del	p.A5_A80del	Nuclear	Negative
HB-11	HB		Not analyzable	r.14_241del	p.A5_A80del	Nuclear	Negative
HB-19	HB	LIT1	None	±215 bp del	p.?	Nuclear	Negative
HB-23	HB		None			Nuclear	Membrane only
HB-24	HB		None	r.14_241del	p.A5_A80del	Nuclear	Negative
HB-27	HB		None			Nuclear	Membrane only
HB-30	HB		c.98_238del	r.98_238del	p.G34_A80del	Nuclear	Negative
HB-32	HB	NF1	c.14-202_241+69del	r.14_241del	p.A5_A80del	Nuclear	Negative

FIGURE 1 *CTNNB1* mutational status and β-catenin IHC pattern of pediatric liver cancers. At large, four different staining patterns were observed using the C-terminal and S33/37/T43 β-catenin antibodies. Combined with the genomic mutation analysis, the most likely β-catenin-related mechanism contributing to tumor formation is color-coded as follows: Grey = no evidence of mutations leading to β-catenin activation. Green = APC mutation or β-catenin D32/S33 amino acid alterations. Blue = β-catenin G34 amino acid alteration. Red = β-catenin exon 3 deletion. "Nuclear" means a combination of nuclear, cytoplasmic, and membranous staining. "Negative" means no staining visible in tumor cells. HCA, hepatocellular adenoma; HB, hepatoblastoma; HCC, hepatocellular carcinoma; FAP, familial adenomatous polyposis; LFS, Li–Fraumeni syndrome; LIT1, LIT1 hypomethylation; NF1, neurofibromatosis type-1; IHC, immunohistochemistry

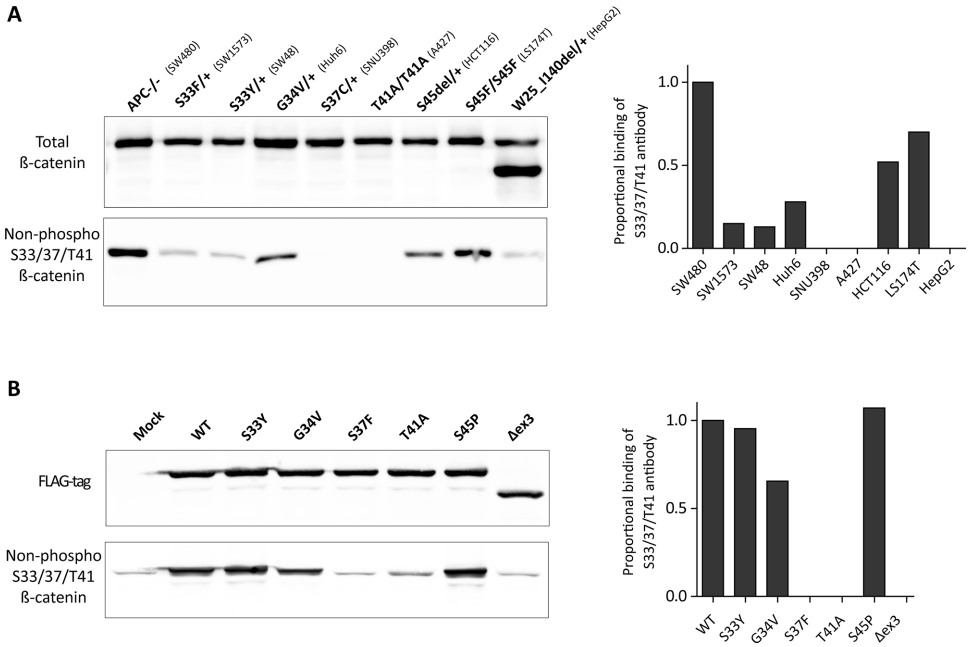

FIGURE 2 Western blot validation of the nonphospho S33/37/T41 β-catenin antibody. (A) Western blot analysis of one APC mutant and eight β-catenin mutant cell lines using a C-terminal β-catenin antibody (upper lanes) in comparison with one raised against a peptide corresponding to residues surrounding S37 (lower lanes). Amino acid alterations and zygosity status are depicted on top. Name of cell line is in brackets. Deletion of exon 3 and alterations of S37 and T41 abrogate binding. Reduced signal for S33 and G34 variants suggests lowered affinity, although residual binding to remaining wild-type protein cannot be formally excluded. S45 changes are largely unaffected. Proportional binding of the S33/37/T41 antibody is shown on the right with the ratio of the *APC*-mutant SW480 cell line set arbitrarily to one. (B) Transient expression of FLAG-tagged wild-type and S/T mutant β-catenin variants shows that S33Y β-catenin can still be recognized by the S33/37/T41 antibody. The G34V variant shows a ±40% reduced signal intensity. The other variants confirm the cell line analysis. The weaker band with lower molecular weight visible in all lanes is the endogenous β-catenin protein produced by HEK-293 cells. Proportional binding of the S33/37/T41 antibody is shown on the right with the ratio of the wild-type protein set arbitrarily to one

3.4 | Immunohistochemical identification of β-catenin alterations

Given the poor quality of DNA extracted from FFPE material, we sought an alternative method to identify samples with genomic *CTNNB1* deletions. All these mutations generate mutant β-catenin proteins that are more resistant to proteolytic breakdown. Hence, the great majority of β-catenin protein present within a *CTNNB1* mutant cell is represented by the mutant protein. This will especially be the case for the nuclear compartment as most of the wild-type protein is prevented from entering the nucleus through degradation by the APC/AXIN breakdown complex. We hypothesized that an immunohistochemical analysis using a β-catenin antibody specifically recognizing the N-terminal S/T residues could distinguish between APC- and β-catenin mutant tumors. In APC-mutant tumors, wild-type β-catenin accumulates in the nucleus that can still be detected by such an antibody, whereas (partial) deletion or mutation of the β-catenin S/T residues would prevent detection.

To validate this approach, we first tested a panel of cell lines with known *CTNNB1* mutations using a C-terminal β-catenin antibody in comparison with one raised against a peptide corresponding to residues surrounding S37. Western blot analysis using the C-terminal antibody robustly demonstrated β-catenin in all cell lines, including the shortened mutant product present in HepG2 cells (Figure 2A). The nonphospho S33/37/T41 antibody readily detected the wild-type β-catenin present in the APC-mutant line SW480. Also the S45 mutant β-catenin present in HCT116 and LS174T was demonstrated at high intensity compared with the C-terminal antibody. In contrast, the exon 3 deleted product of HepG2 and S37/T41 alterations completely abrogated detection, while S33 and G34 alterations resulted in reduced detection. The latter may represent binding to wild-type β-catenin present in these cells and/or retained affinity for the mutant protein.

To investigate residual binding affinity for mutant β-catenin by the S33/37/T41 antibody, we transiently expressed N-terminal FLAG-tagged β-catenin variants in HEK-293 cells (Figure 2B). Deletion of exon 3, and S37F/T41A alterations completely abrogated binding. In contrast, the S33Y variant was identified at comparable levels to the wild-type protein, while the G34V variant is detected with ±40% reduced signal intensity. In conclusion, the S33/37/T41 antibody retains affinity for S33/G34 alterations when present in denatured form on western blot, while S37/T41 changes and deletion of exon 3 prevent its binding.

Next, we evaluated this antibody for immunohistochemical purposes using paraffin blocks containing formalin-fixed β-catenin mutant cell lines. In line with the western blot data, S45 mutant HCT116 and LS174T showed a comparable staining pattern for both antibodies (Figure 3). In strong contrast, all other β-catenin mutant lines showed

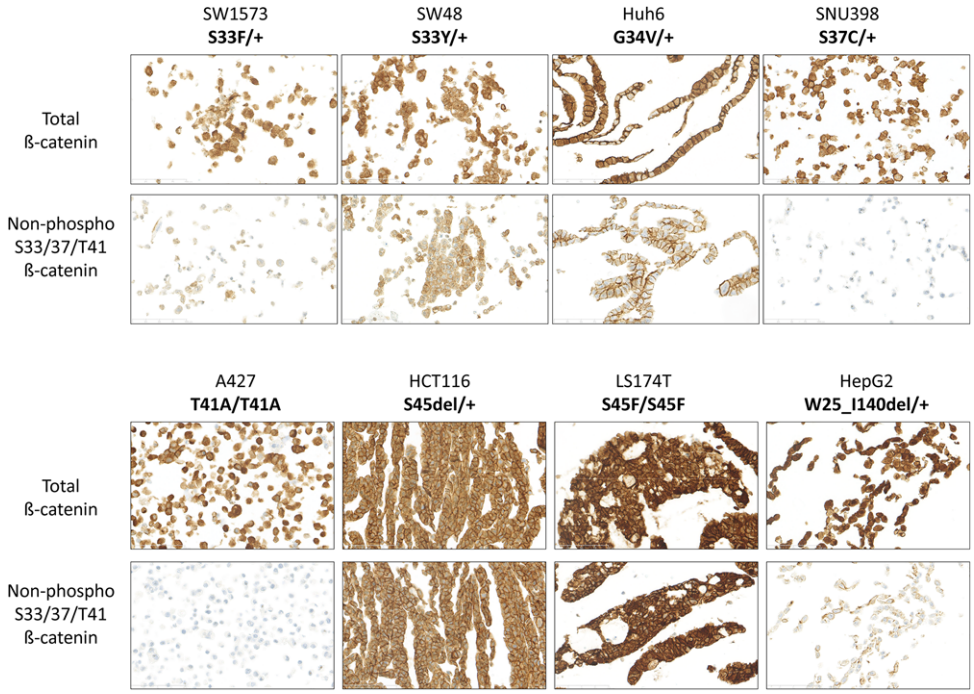

FIGURE 3 Immunohistochemical validation of the nonphospho S33/37/T41 β-catenin antibody. Formalin-fixed and paraffin-embedded cell pellets of the β-catenin mutant cell lines were stained with the C-terminal (top rows) and S33/37/T41 (bottom rows) β-catenin antibodies. Staining patterns are largely in line with western blot analysis with the exception of the relatively weak staining pattern observed for the G34V protein present in Huh6. HepG2 shows an exclusive membranous staining with the S33/37/T41 antibody, most likely reflecting the remaining wild-type protein. Both S33 mutant cell lines retain some nuclear detection, albeit slightly reduced. S45 variant cell lines show identical staining patterns with both antibodies. Original magnification 400×

a clearly reduced overall staining using the S33/37/T41 antibody. The S37C and T41A mutant lines are completely negative. In the S33 mutant lines SW48 and SW1573 nuclear staining can be observed in some cells, although with reduced intensity. The exon 3 deletion mutant HepG2 line showed an exclusive membranous staining, most likely resulting from detection of the remaining wild-type protein. Also in the G34V mutant Huh6 line no obvious nuclear staining was observed. Surprisingly, IHC staining of this variant is much weaker than anticipated from the western blot analysis, most likely resulting from reduced antibody affinity when present in its "native" form in FFPE sections and/or partial phosphorylation of S37/T41 residues. In conclusion, the S33/37/T41 β-catenin antibody can reliably identify the cell lines with exon 3 *CTNNB1* mutations, with the exception of S33 and S45 alterations.

Next, the S33/37/T41 antibody was applied to the tumor sections and compared with that of total β-catenin (Figure 4, Supplementary Figure S1). At large, four different staining patterns were observed. The tumors with exclusively membranous β-catenin were stained in an identical fashion with the S33/37/T41 antibody (Figure 4A and 4B, Supplementary Figure S1). Importantly, all three FAP-associated lesions showed basically identical staining patterns with both antibodies as well, that is, a high proportion of tumor cells showing evidence of nuclear accumulation (Figure 4C and 4D, Supplementary Figure S1). This pattern was also observed in lesions with D32 and S33 mutations, albeit with slightly lower intensity for the S33/37/T41 antibody (Figure 4E and 4F, Supplementary Figure S1). The third pattern is observed in HBs with G34V mutation. These showed a strongly reduced overall staining using the S33/37/T41 antibody. However, occasionally tumor cells with nuclear staining can be clearly observed, especially in regions with heavy staining for total β-catenin (Figure 4G and 4H, Supplementary Figure S1). This pattern was also observed in HB-28, the only lesion with a T41 mutation, in which a heavy overall β-catenin staining is accompanied by occasional nuclear-positive cells using the S33/37/T41 antibody (Supplementary Figure S1). As the T41A alteration cannot be detected by this latter antibody, the nuclear staining most likely results from wild-type protein induced to enter the nucleus. Lastly, in both HBs with proven genomic exon 3 deletions virtually no tumor cell staining was detectable (Figure 4I and 4J, Supplementary Figure S1). A similar pattern was also identified in six additional samples with no identifiable point mutation, suggesting that, in total, seven out of 18 HB samples and one HCC sample carry genomic exon 3 deletions. In two of these samples, some residual membranous staining was visible (HB-23 and HB-27, Supplementary Figure S1).

3.5 | Confirmation of exon 3 deletion by RT-PCR

To confirm that these latter samples indeed express mutant *CTNNB1* mRNA (partially) lacking exon 3, we performed RT-PCR on RNA isolated from paraffin sections. Successful RNA isolation was possible in six samples. In four out of six samples, a 127 bp product was observed indicative of a complete absence of exon 3 (Figure 5). In sample HB-30 a shortened 214 bp product was observed, which, after sequencing, was confirmed to carry the same intra-exonic 141 bp deletion already

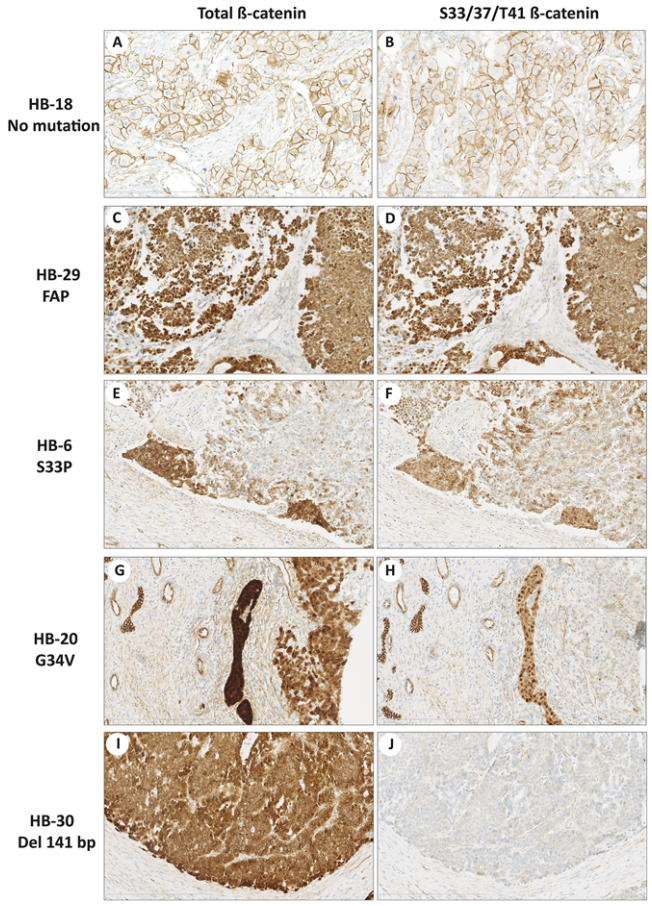

FIGURE 4 Immunohistochemical identification of β-catenin alterations in tumor samples. FFPE sections of pediatric liver tumors were stained with the C-terminal (left column) and S33/37/T41 (right column) β-catenin antibodies. (A,B) Comparable membranous staining in the fibrolamellar hepatocellular carcinoma (HB-18) with no evidence of β-catenin mutation or activation. (C,D) HB-29 derived from a FAP patient shows identical nuclear and cytoplasmic accumulation of wild-type β-catenin using both antibodies. (E,F) In sample HB-6 a p.S33P mutation was detected, which shows nuclear staining with both antibodies, albeit slightly weaker using the S33/37/T41 antibody. (G,H) Sample HB-20 carrying a p.G34V mutation shows clear nuclear β-catenin accumulation in tumor sections. Staining with the S33/37/T41 antibody is strongly reduced or entirely lost in tumor cells, while normal structures on the left are stained in an identical fashion. (I–L) The strong nuclear and cytoplasmic staining of sample HB-30 with genomic deletion of exon 3 is completely lost using the S33/37/T41 antibody. Original magnification 200×

observed during the genomic DNA analysis. Sample HB-19 showed a shortened reproducible product of about 140 bp, indicating that an approximate 215 bp deletion is present in the cDNA. In conclusion, all six samples with negative S33/37/T41 β-catenin nuclear staining and absence of exon 3 point mutation showed evidence of (partial) exon 3 deletion on RNA level.

Overall, our combined IHC, RNA, and DNA analysis shows that all 18 HBs are characterized by increased β-catenin signaling. In three samples, the underlying cause is a germline *APC* mutation, while activating *CTNNB1* point mutations are identified in eight cases. For another five HB cases, we provide evidence that they carry genomic

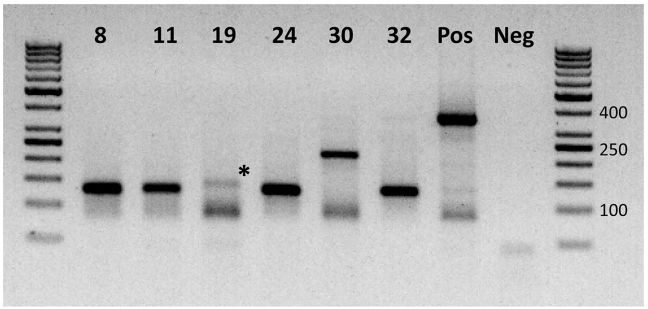

FIGURE 5 Confirmation of exon 3 deletion by RT-PCR. RNA was isolated from six HBs with immunohistochemical evidence of (partial) exon 3 loss, and subjected to RT-PCR using primers in exon 2 and 4. In four out of six samples, a 127 bp product was observed indicative of a complete absence of exon 3 (HB-8, HB-11, HB-24, and HB-32). Sample HB-19 showed a reproducible shortened product of approximately 140 bp (*). In sample HB-30, the intra-exonic 141 bp genomic deletion was confirmed at RNA level. Positive control is RNA isolated from normal tissue

exon 3 deletions; this is also highly suggestive for the remaining two cases based on their IHC staining pattern (HB-23 and HB-27).

4 | DISCUSSION

Most HBs are believed to be of sporadic nature, but about 10–20% have been associated with genetic defects such as Beckwith–Wiedemann syndrome or FAP.[2,5,6] For this reason, it has been advocated that all HB patients with unknown family history should be referred for genetic counseling.[6] Among the genetic syndromes associated with HB development, FAP is one of the most common with an estimated frequency between 5 and 10%.[2,16] As this is a severe disease, it is important for the patient and also for yet asymptomatic family members to determine their carrier status at an early stage. Especially a firm exclusion of an underlying *APC* germline mutation will prevent the unexpected emergence of polyposis or colorectal cancer at a later stage. Current diagnosis largely depends on *APC* germline mutation detection on genomic DNA, but inherent to any diagnostic method this will occasionally lead to false-negative results. In case of classical polyposis phenotypes, the *APC* mutation detection rate is around 80–90%,[35,36] meaning that a possible 10–20% of FAP patients may be missed by routine genetic testing. This leads to the highly undesired scenario in which apparent *APC* mutation negative patients develop polyposis later. For this reason, Aretz et al. proposed that all *APC*-negative HB patients undergo colonoscopy around age 15 and one at age 25, to also offer parents the option to undergo colonoscopy.[2] The downside of this approach is that many genuine *APC*-negative individuals will be exposed to unneeded medical investigations associated with anxiety and considerable costs. Here, we establish and validate a tissue-based β-catenin gene and immunohistochemical analysis that complements the germline mutation screening to exclude the diagnosis of FAP among HB patients. Following successful confirmation in an independent series, its application will lead to a near-complete exclusion of FAP among HB patients.

Our method is based on the observation that tumor-driving *CTNNB1* and *APC* mutations occur in a mutually exclusive nature in all tumor types studied so far.[12] As such, the identification of a somatic activating *CTNNB1* mutation in an HB patient directly reduces the risk of carrying a germline *APC* mutation to that of the general population. By combining mutational analysis of *CTNNB1* exon 3 and IHC comparing staining patterns for total and S33/37/T41 β-catenin, we could reliably identify all three FAP patients in our cohort of 18 HB cases. Their tumors are characterized by a comparable nuclear staining for both antibodies and wild-type exon 3 sequence. In fact, the IHC analysis by itself can already select a large proportion of HBs that carry an activating *CTNNB1* mutation, and thus have no increased risk of an underlying *APC* germline mutation. Our epitope and immunohistochemical analysis suggests that all amino acid alterations and genomic deletions encompassing the G34-T41 region would prevent or strongly reduce binding of the S33/37/T41 antibody. Reviewing the literature for the type of *CTNNB1* mutations reported in HBs, it was found that about 85% of mutations (198/234) fulfill this criterion.[13–21] The main exceptions are D32/S33 amino acid alterations, while S45 mutations are rarely observed in HB (3/234). Although the IHC analysis can already identify most HB patients with *CTNNB1* mutations, we nevertheless propose to also include the exon 3 mutation analysis on tumor DNA. This will identify point mutations outside the epitope of the S33/37/T41 antibody (D32, S33, and S45), and provides a confirmation of the *CTNNB1* mutational status.

As *CTNNB1* mutations are the predominant mutation in pediatric liver cancers (up to 80%), most patients are unlikely carriers of a germline *APC* mutation. A small subset of cancers arises without apparent β-catenin activation, that is, the ones with exclusively membranous β-catenin staining and wild-type sequence. As it is highly unlikely that such a β-catenin nuclear-negative tumor arises in a FAP patient, these are also unlikely to carry a germline *APC* mutation. Anecdotally, somatic *APC* and *AXIN1*/*AXIN2* mutations have been reported in HBs that also lead to tumors in which wild-type β-catenin accumulates in the nucleus.[15,16,22] These rare tumors would lead to *APC* germline negative patients, whereas the tumor analysis indicates an underlying FAP syndrome. In the next section, we discuss how to deal with this small subset of HB tumors.

How can our method be implemented in current clinical practice? As HBs can be a manifestation of several genetic syndromes, all patients should be offered genetic counseling and evaluated for syndromic features such as the macrosomia and organomegaly associated with BWS. In the absence of such features and a negative family history, a genetic mutation analysis is warranted. This should include at least the *APC* gene, but may also include other genetic cancer predisposition syndromes such as LFS and NF1.[42,43] Simultaneous with or preceding the counseling process, we propose to combine a routine histopathological evaluation of the tumor tissue with both β-catenin stainings described here. In addition, tumor DNA should be isolated from the FFPE-material and evaluated for *CTNNB1* exon 3 mutations, which can also be done as part of a larger next-generation sequencing panel.

In most cases, the genetic and tumor tissue analyses will corroborate each other. Identification of a germline *APC* mutation will be accompanied by a comparable staining with both antibodies and wild-type *CTNNB1* sequence in the tumor tissue, while *APC* germline negative cases will show somatic activating *CTNNB1* mutations and reduced staining with the S33/37/T41 antibody. However, given the approximate 10–20% false-negative *APC* mutation analyses, occasionally tumors will be identified showing nuclear accumulation of wild-type β-catenin without an identifiable *APC* germline mutation. As these cases are at increased risk to develop FAP at a later stage, colonoscopies of the index patient and its parents can be performed, as suggested by Aretz et al.[2] Using our diagnostic method, this can however be restricted to the subset of *APC* germline negative patients that express wild-type β-catenin in the tumor. Alternatively, the somatic mutation analysis of these tumors can be extended to identify inactivating somatic *AXIN1/2* or *APC* mutations; when *AXIN1* or *AXIN2* mutations are identified, this also excludes the diagnosis FAP. In the case of a "somatic" *APC* mutation, more caution is needed as this may indicate a missed germline mutation that is present, for example in a low-mosaic fashion in the patient.

Our approach can also be extended to other extracolonic FAP manifestations, such as pilomatricomas and desmoids. These lesions are also characterized by high frequencies of somatic β-catenin mutations, meaning that most are of sporadic nature.[44–50] About one-third of *CTNNB1* mutations reported for desmoids are at codon S45, and are missed by the IHC method described here, but—if desired—this analysis can be extended by including an antibody specifically recognizing this epitope (e.g. #19807, Cell Signaling technology).

In conclusion, we have successfully established and validated a novel ß-catenin genetic and immunohistochemical diagnostic method, which—following confirmation in an independent series—allows the near-complete exclusion of the diagnosis of FAP among HB patients, when combined with routine germline DNA testing.

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AUTHOR CONTRIBUTIONS

H.J.D., I.H.I.M.H., M.M.v.N, W.N.M.D., A.W., and R.S. designed research. I.H.I.M.H., C.A.V., W.W., P.L., and M.D. acquired data. All authors analyzed and interpreted the data. All authors were involved in writing the paper and had final approval of the submitted and published versions.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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SUPPORTING INFORMATION

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