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Article

Mutual Tissue Microchimerism of Hepatoblastomas in Monozygotic Twins From a Familial Adenomatous Polyposis Family

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Abstract

Patients with familial adenomatous polyposis (FAP) have increased risk of hepatoblastoma (HB). We report monozygotic twins with HB in a FAP family. To explore genetic alterations in the HBs of the twins, we carried out whole exome sequencing (WES), RNA-seq, and immunohistochemical analyses of the tumors. Additional multiregional digital PCR was performed to profile clonality of each tumor. To determine a pathogenic germline variant in *APC*, Sanger sequencing was applied for the twins, the father, and the siblings of the father. A pathogenic variant of the *APC* gene was identified in the father as well as the twins. The WES of the HBs in the twins identified somatic mutations, including an *NRAS* mutation in the tumor of the first infant (C1), and an *ACVR2A* mutation in the tumor of the second infant (C2). No somatic mutations were identified in the genes associated with the Wnt signaling pathway. However, accumulation of β-catenin was found in the C1 and C2 tumors by immunohistochemical staining, suggesting activation of the Wnt signaling pathway. Digital PCR analysis revealed that the *NRAS* mutation was found in multiregional specimens of C1 and those of C2. The *ACVR2A* mutation was found in multiregional specimens of C2, whereas the mutation was also identified in those of C1. The existence of a shared somatic mutation may suggest that microchimerism took place in the development of HBs through the utero-placental circulatory system. Importantly, the initiation of tumorigenesis is thought to occur during the fetal period after organ development of the liver.

Keywords: Hepatoblastoma; Germline mutation; APC; Monozygotic twins; Familial adenomatous polyposis

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Hepatoblastoma (HB) is the most common form of hepatic malignant tumors in children. While several genetic and epidemiologic studies of HB have been conducted, the molecular mechanisms underlying HB in FAP patients have not been fully elucidated. The majority (70%) of HB cases are sporadic and the number of genetic alterations is known to be relatively small compared to adult solid tumors (Eichenmuller et al., 2014; Jia et al., 2014; Nagae et al., 2021). Most HBs are found in infants, for which hepatectomy and/or chemotherapy is considered as standard therapy (Hiyama et al., 2020; Katzenstein et al., 2019; Perilongo et al., 2009; Zsiros et al., 2013). If the histology is pure fetal type, then the prognosis is favorable (Malogolowkin et al., 2011).

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Among the limited number of genetic changes, mutations of β-catenin (*CTNNB1*) have been most frequently observed, followed by *APC* and *LGR6* (Perugorria et al., 2019) in HB. Importantly, it has been reported that the risk of HB is 750 to 7500-fold higher in FAP individuals who carry pathogenic germline variants (Kinzler et al., 1991; Nishisho et al., 1991) than in the general population from a population-based registry (Aretz et al., 2006; Giardiello et al., 1991; Giardiello et al., 1996; Hughes & Michels, 1992). These data indicate that abrogation of the Wnt signaling pathway plays a crucial role in HB development (Evers et al., 2012; Flanagan et al., 2019; Giardiello et al., 1996; Perugorria et al., 2019; Thomas et al., 2003).

Here we report monozygotic twins from a FAP family who suffered from HB asynchronously. Whole exome sequencing and subsequent analysis of their tumors uncovered that microchimerism might occur during the development of HB at a very early stage of life.

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Materials and Methods

Clinicopathological Data and Tumor Specimens

A family tree was constructed using a free online pedigree tool. Posttherapeutic (preoperational chemotherapy) specimens (C1) were obtained from Infant-1, whereas only postchemotherapeutic/operational specimens (C2) were obtained from Infant-2 since an emergency operation was performed due to signs of severe hemorrhage during preoperative chemotherapy. The DNA from histologically confirmed tumors were extracted using either a GeneRead DNA FFPE kit (Qiagen, Hilden, Germany) or QIAmp DNA Mini Kit (Qiagen). The tumor cellularity of each tumor specimen was estimated by a certified pathologist (K.I.).

Germline Mutation Analysis

Genomic DNA was extracted from either peripheral blood mononuclear cells (PBMCs) or oral mucosal samples from the twins, their parents, and all siblings (three sisters) of the father using the standard phenol extraction/purification procedure (Sato et al., 1990). The coding exons in *APC* were amplified with M13-tailed target-specific primers and the PCR products were sequenced on an Applied Biosystems 3730xl DNA Analyzer using the BigDye Direct Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA) (Yamaguchi et al., 2016). The primer sequences are available upon request.

Whole Exome Sequencing (WES)

For WES, we isolated DNA located in protein-coding exons using SureSelect Human All Exon v6 kits according to the manufacturer's instructions (Agilent Technology, Santa Clara, CA). Captured DNA fragments were sequenced using a HiSeq 2500 (Illumina, San Diego, CA) with a standard 125 bp-paired end read protocol. Reads mapping and further analyses were performed using Genomon2 (v.2.6.3), which is an in-house pipeline constructed at the Human Genome Center based at The University of Tokyo (https://github.com/Genomon/genomon). Briefly, sequence reads were aligned to the human reference genome hg19 using Burrows–Wheeler Aligner-MEM (v.0.5.10) and bam files were created for data processing. For the Genomon2 pipeline analysis, the default settings were used for each parameter, and the EBCall (Empirical Baysian mutation Calling) algorithm was used to discriminate somatic mutations from sequencing errors (Shiraishi et al., 2013). Finally, SNVs (single nucleotide variants) and short indels were identified using the following procedure: (1) remove reads resulting from strand-specific sequencing errors ($0.2 \leq$ 'strandRatio_tumor' ≤ 0.8); (2) remove intronic and UTR (untranslated region) variants; (3) read depth 'depth_tumor/normal' ≥ 50; (4) variant allele frequency 'misRate_tumor' ≥ 0.05; and (5) manual review of the aligned reads using the Integrative Genomics Viewer (IGV).

Molecular Assessment of the Wnt Signaling Pathway

RNA-seq analysis was performed using RNA extracted from the tumorous and nontumorous liver tissues in C2. RNA was extracted from the tissues using RNeasy Plus Mini Kit (Qiagen), and the quality and quantity of RNA were calculated using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA sequencing libraries were prepared using the TruSeq RNA Access Library Prep Kit according to the manufacturer's protocol (Illumina). Sequencing was performed with paired-end reads of 100 bp on an Illumina HiSeq 2500 platform. Read alignment and counting were performed using GenomonExpression (v.0.5.0), which is an in-house pipeline (https://github.com/Genomon-Project/GenomonExpression).

The expression of β-catenin in HBs was evaluated by immunohistochemical staining using an anti-β-catenin antibody (#9582, Cell Signaling Technology, Danvers, MA). The sections were deparaffinized with xylene. After antigen retrieval (10 mM sodium citrate buffer for 10 min at 115°C), non-specific binding was blocked with goat serum, followed by overnight incubation in the antibodies (dilution 1:200) at 4°C. After washing, the tissue-antibody reaction was visualized using the EnVision+ System (Agilent Technologies). Hematoxylin was used for nuclear counterstaining.

Digital PCR

Variant allele frequency (VAF) of somatic mutations in tissue and plasma were quantified by means of digital PCR (dPCR). Specific primer pairs of mutations identified by WES from tumors were designed and obtained from OTS-Probes, which is a dPCR probe library that increases the primer and probe annealing specificity with modified bases for amplification of short fragments (Quantdetect Inc., Tokyo, Japan). The probe sequence was labeled either by FAM or VIC fluorescent dyes for wild and mutant types, respectively. Both dyes were quenched by a Blackhole quencher (BQ) dye. The extracted DNA from FFPE and infant plasma were subjected to dPCR reactions in a QuantStudio 3D system (Thermno) and detected with originally developed primer/probe sets (Iwaya et al., 2021) (Quantdetect).

Results

Family History

Although two tumor-related deaths and colon polyps were noted in the pedigree, there was no previous history of HB in any family members except for the twins (Figure 1A). All tumor-related lesions were from gastrointestinal origins, including colon and gastric cancer. The paternal grandfather (II-1) passed away due to a traffic accident, but he had been previously treated for colon polyps. The father (III-6) developed gastric and colon cancer in his 20s and underwent curative surgery for these tumors. Periodic follow-up examinations revealed no recurrent disease for the past 7 years.

Germline Mutations

We carried out genetic analysis of the father's DNA and identified a 7-base pair-deletion (NM_000038.6, c.3231_3237delTTATACT (p. Val1077fs) in the coding region of *APC*. Located in the β-catenin binding region of APC, this variation is predicted to cause a frameshift, leading to the production of truncated APC protein lacking the β-catenin binding region (Figure 1B). Subsequent cascade analysis identified the variant in the twins but not in the mother or father's siblings by Sanger sequencing (Figure 1A, 1C).

Clinical Course of Infant-1 (C1 Tumor)

The twins were born by cesarean section from a 28-year-old mother at 38 weeks gestation. No parental or neonatal problems occurred during the delivery course. They were clinically dichorionic-diamniotic gestational twins. The birth weight of the first baby was 2594 g and that of the second was 2326 g (Figure 2A and 2B). The infants were

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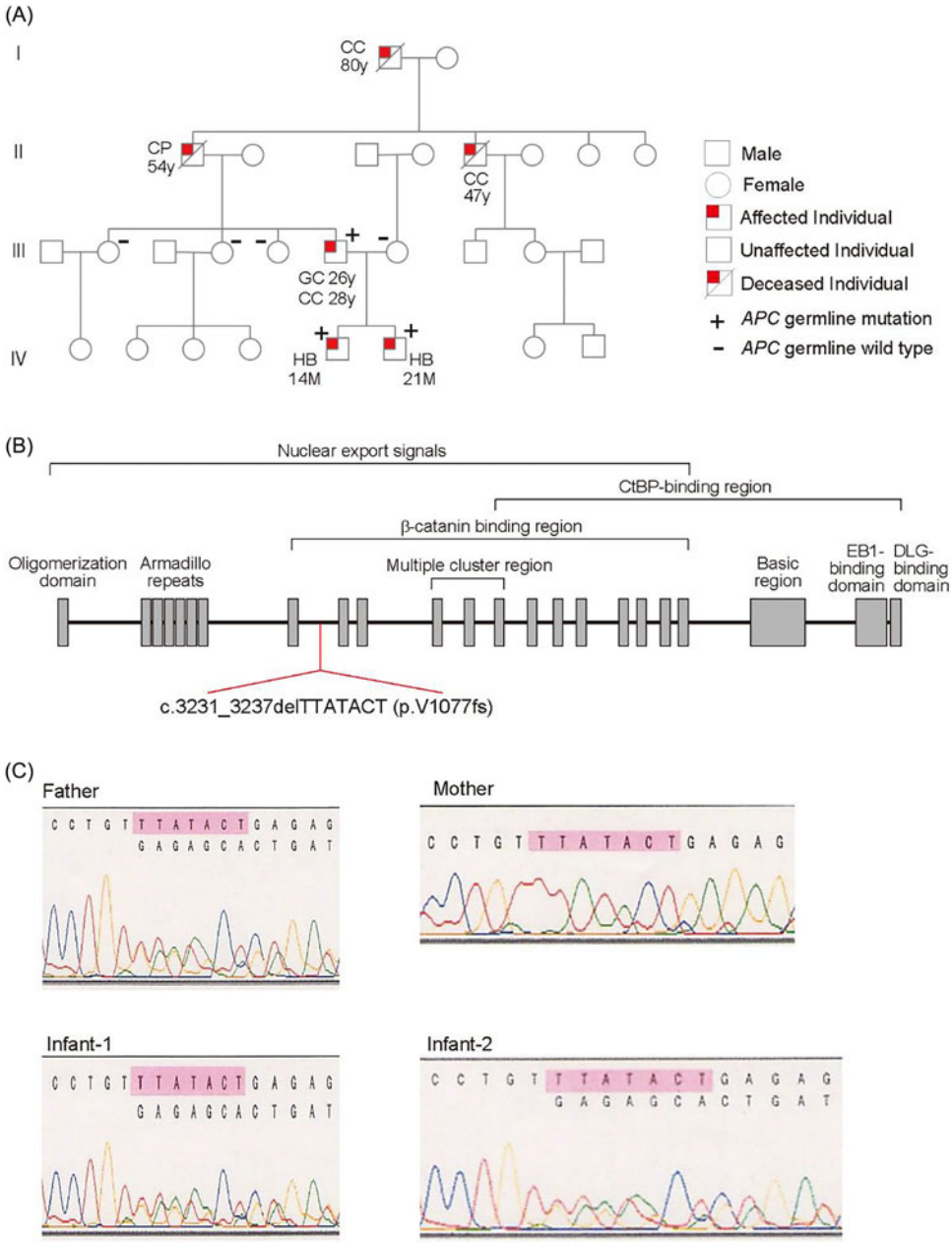

Figure 1. Genetic background of the twins. (A) Family pedigree. (B) Structure of the APC gene, including functional domains and introns. The position of the identified germline mutation is indicated by the red line. (C) Sanger sequence histogram of the affected infants and their parents.
Note: CC, colon cancer; CP, colon polyps; GC, gastric cancer; HB, hepatoblastoma.

discharged in stable condition and exhibited a normal developmental course. However, the first of the twins gradually lost appetite and suffered abdominal distention by 12 months of age (Figure 2A). The parents took him to our hospital for consultation of his symptoms at 14 months of age. Physical examinations showed abdominal distention, particularly in the right quarter rib, where an elastic soft mass with the size of approximately 14 cm was palpable. An ultrasound examination detected a liver mass with the consistent size of 14 cm. Because of hyperechoic and nonuniform features of the mass content as well as the size, hepatoblastoma was suspected. Additional computed tomography (CT) scans showed a large, round, and smooth liver mass whose internal density was not uniform, with apparent invasion to the hilar plate that possibly originated from the posterior segment (PRETEXT I) (Brown et al., 2000). Blood laboratory examinations revealed slight elevation of white blood cells (14,600 / mL), anemia (Hb 8.4 g/dl), and a high level of AFP (33,961 ng/ml), which is a tumor marker of hepatoblastoma. A pathological examination of the tumor biopsy revealed that the tumor histology was a pure fetal type of HB (Supplementary Figure S1A). Based on this diagnosis, two cycles of preoperative chemotherapy (cisplatin/pirarubicin, CITA) were performed, which resulted in a marked reduction of the tumor volume to 9 cm in diameter. A right lobe hepatectomy was subsequently performed at 17 months of age. The surgical margin was negative for cancer and no detectable metastases to other organs were found in his abdominal cavity. The patient had a good postoperative course and underwent adjuvant chemotherapy 20 days after the surgery with one cycle of CITA followed by three cycles of low-CITA. No evidence of metastasis or recurrence has been observed for 7 years since the completion of adjuvant chemotherapy.

Clinical Course of Infant-2 (C2 Tumor)

Since the first of the twins (Infant-1) was diagnosed with HB, Infant-2 underwent an abdominal ultrasound examination for

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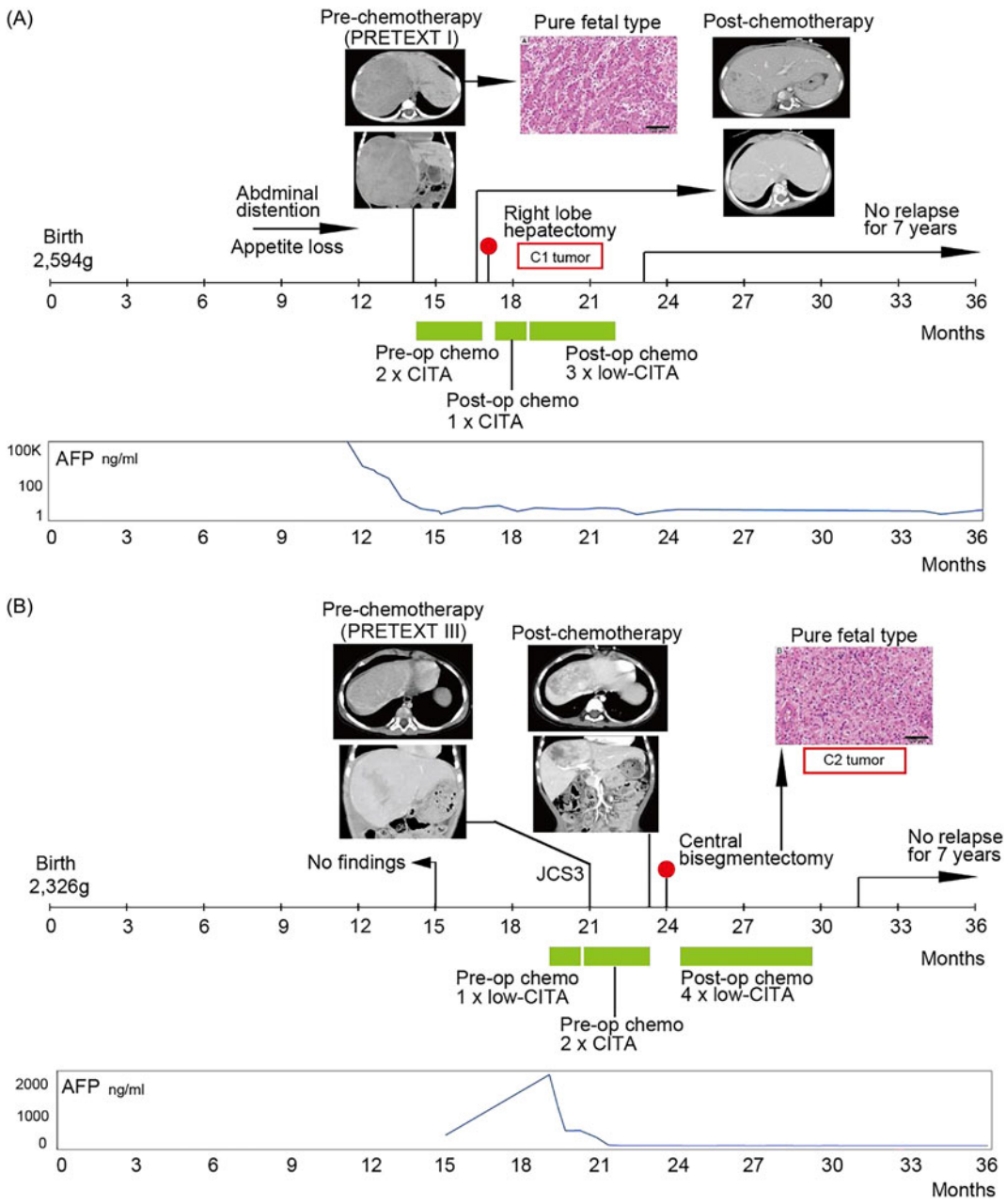

Figure 2. Clinical course of the affected infants. The C1 tumor was obtained by biopsy, whereas the C2 tumor was obtained by hepatectomy. (A) Infant-1. The red circle indicates the date of hepatectomy. (B) Infant-2. The red circle indicates the date of hepatectomy.

HB screening at 15 months of age (Figure 2B) despite not having any symptoms. No neoplastic lesions were detected in his liver at the time of screening. However, he was transported to the emergency department in our hospital at 21 months of age due to a sudden onset of respiratory distress with pallor on his face and subsequent loss of consciousness within a day. On admission, a pallor face and acrocyanosis of the extremities were noticed, and he suffered grade 3 unconsciousness based on the Japan Coma Scale (not awake with external stimulation). In addition, overall abdominal distention was observed, and physical examination detected a palpable and elastic soft mass on his right upper quadrant. Blood laboratory examinations showed a slight increase in the number of white blood cells (12,580 /μL), severe anemia (Hb, 6.9 g/dL), and moderately high AFP (2,127 ng/mL). An emergency CT scan demonstrated that his liver was occupied by a large mass with a diameter of 11 cm that had clear and smooth margins, a capsule-like structure, and heterogenous intensity. The CT scan images additionally depicted a low-density area at the center of the tumor, which strongly indicated tumor hemorrhage. Therefore, we did not perform a tumor biopsy for pathological diagnosis, but instead made a PRETEXT III diagnosis of suspected HB[23] according to the images. To manage tumor removal without trisectionectomy, preoperative chemotherapy was given, including one cycle of low-CITA followed by two cycles of CITA. Cycles of the chemotherapy reduced the tumor volume down to 6.6 cm in diameter without additional major hemorrhage from the tumor. The patient then underwent a central bisegmentectomy of the liver at 24 months of age. Pathological examination of surgical specimens confirmed that the tumor was the pure fetal type of HB (Supplementary Figure S1B), which was the same histological type as his brother. Twenty days after hepatectomy, four cycles of CITA postoperative

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Table 1. Representative somatic mutations that may serve as tumor development trackers

					Tumor			Normal				
Sample	Gene	Nucleotide change	Amino acid change	Cytoband	Variant read	Total read	VAF	Variant read	Total read	VAF	InterVar	COSMIC85
C1	*NRAS*	c.35G>A	p. Gly12Asp	1p13.2	9	179	5.0%	0	226	0%	LP	COSM564
C2	*ACVR2A*	c.16A>T	p. Lys6Ter	2q22.3	92	643	14.2%	0	136	0%	US	NA

Note: VAF, variant allele frequency; InterVar, https://wintervar.wglab.org/about.php; LP, Likely pathogenic; US, Uncertain significance; COSMIC, Catalogue of Somatic Mutations in Cancer database; NA, Not applicable.

chemotherapy were initiated. No evidence of recurrence or metastasis has been observed over the 7 years since the surgery.

Involvement of Wnt Signaling Pathway in Tumor Development

An immunohistochemical evaluation of the β-catenin protein was performed on the tumor tissue. Both tumors (C1 and C2 tumors) showed positive staining in the cytoplasm of HB cells, but not adjacent normal cells (Supplementary Figure S2). Nuclear localization was occasionally seen in the C1 tumor, but was clearly evident in the C2 tumor. In addition, an RNA-seq analysis of the C2 tumor and the corresponding nontumorous tissues revealed that expression of *ODAM*, *LGR5*, and *AXIN2*, which are three direct target genes in the Wnt pathway, was markedly augmented in the tumor tissue compared to the nontumorous tissue. These data suggest that activation of the Wnt signaling pathway was involved in the development of the HB tumors.

Genetic Analysis of the HBs

We carried out WES on the tumor tissues of the twins (Infant-1 and Infant-2) and nontumorous DNA extracted from their peripheral blood. The average sequence coverage of each exon was 148 (Infant-1) and 182 (Infant-2) for the nontumorous DNA, and 150 (C1) and 196 (C2) for the tumor tissue respectively. Comparison of the germline SNVs between Infant-1 and Infant-2 confirmed that the twins were monozygotic.

We identified a total of eight and one somatic SNVs in C1 and C2 respectively. In the eight SNVs in C1, six were nonsynonymous variants, one was a synonymous variant, and the remaining one was a nonsense mutation. The SNV in C2 was a nonsense mutation.

No somatic mutation was shared in the two tumors. As for driver mutations, we identified an *NRAS* mutation, NM_002524.5, c.35G>A (p. Gly12Asp) in C1 (with a VAF of 7.1%), and an *ACVR2A* mutation, NM_001616.5, c.16A>T (p. Lys6Ter) in C2 (with a VAF of 14.2%) (Table 1).

To determine whether the mutations were a late event in the evolution of each HB, we carried out multiregional analysis of the *NRAS* and *ACVR2A* mutations by digital PCR analysis. DNA were extracted from six regions of the C1 tumor and seven regions from the C2 tumor, and the mutations were determined using the mutant-specific probe sets. As shown in Figure 3A, the *NRAS* mutation was detected with different frequencies; 0.2% (region-1), 0.03% (region-2), 0.02% (region-3), 0.2% (region-4), 0.16% (region-5), and 0.14% (region-6) in the C1 tumor, implying intratumorous heterogeneity. Surprisingly, we also found the *NRAS* mutation in the seven regions of the C2 tumor with the following frequencies: 0.13% (region-5), 0.16% (region-6), 0.09% (region-7), 0.18% (region-8), 0.10% (region-10), 0.09% (region-11), and 0.11% (region-12) (Figure 3B). Similarly, the *ACVR2A* mutation was identified not only in the C2 tumor (0.42% in region-5, 0.19% in region-6, 2.24% in region-7, 4.06% in region-8, 7.10% in region-10, 1.10% in region-11, and 2.73% in region-12), but also in four of the six regions of the C1 tumor (0.03% in region-1, 0.02% in region-2, 0.4% in region-4, and 0.03% in region-6; Figure 3A and 3B). It is extremely unlikely that the two identical somatic mutations occurred in independent tumor clones after birth. Hence, these results suggest that: (1) implantation of tumor clone(s) occurred through utero-placental blood circulation systems, giving rise to microchimerism of both tumors; and (2) the initiation of HB tumorigenesis likely occurred during the fetal stage after organ development of the liver.

Circulating Tumor DNAs

To investigate the usefulness of the driver mutations for their surveillance, we tested their detection in the ctDNA. Digital PCR analysis identified the *ACVR2A* mutation in prechemotherapeutic plasma of Infant-2 at a VAF of 1.31%, but not in postoperative plasma. As expected, the *NRAS* mutation was not detected in postoperative plasma of Infant-1 (no preoperative plasma available). These mutations were not detected in DNA extracted from PBMCs of both infants. Neither mutation was detected in the parents' plasma DNA (Table 2).

Discussion

HB is a rare embryonal malignant tumor arising from the liver yet is the most common malignant tumor in infants and children. It commonly occurs in the first few years of life and predominantly in boys (Giardiello et al., 1991; Giardiello et al., 1996; Trobaugh-Lotrario et al., 2018). FAP is an autosomal dominant disorder forming thousands of colorectal adenomas during adolescence and young adulthood, which is caused by a germline variant of the *APC* tumor suppressor gene. Although clinical characteristics of HB-affected individuals with *APC* mutations do not seem to be distinct from those with sporadic HB, it has been estimated that individuals with a FAP background have a 750 to 7500-fold higher risk of developing HB than the general population (Aretz et al., 2006; Giardiello et al., 1996; Hughes & Michels, 1992). Although investigators have reported *APC* mutations in HB patients (Aretz et al., 2006; Evers et al., 2012; Gupta et al., 2013; Ponz de Leon et al., 2014; Thomas et al., 2003; Yang et al., 2018), a concomitant genetic analysis of HB, or HB from twins in FAP pedigrees, has not been fully performed.

It has been reported that HBs have fewer somatic mutations at the whole-genome level than most adult tumors do, including those of adult hepatocellular carcinomas (Eichenmuller et al., 2014; Fujimoto et al., 2016; Jia et al., 2014; Nagae et al., 2021). The activation of the Wnt signaling pathway with an alteration of *CTNNB1* has been suggested to be the genetic event observed most

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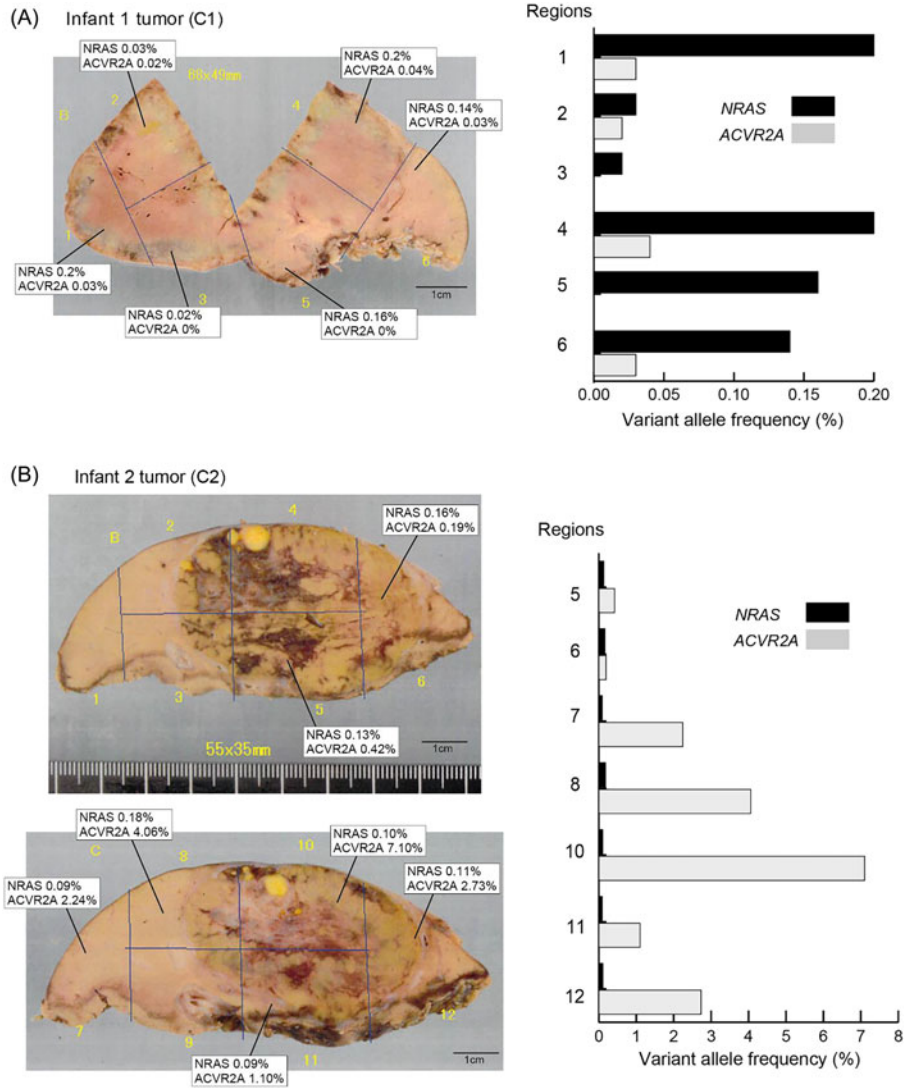

Figure 3. Rare variant detection from multiregional specimens. (A) A macroscopic slice of the Infant-1 tumor (C1). DNA was extracted from six different regions. The variant allele frequency (VAF) of *NRAS*/*ACVR2A* in each region is indicated in a bar graph. (B) Macroscopic slices of the Infant-2 tumor (C2). DNA was extracted from seven different regions. The VAF of *NRAS*/*ACVR2A* in each region is indicated in a bar graph.

Table 2. Variant allele frequencies (%) of ctDNA

				Plasma					
Individuals	Gene	Nucleotide change	Amino acid change	At rest	Postchemo (prehepatectomy)	Prehepatectomy	Posthepatectomy	Postchemo (posthepatectomy)	PBMC
Father	*NRAS*	c.35G>A	p. Gly12Asp	0 (0/183)[a]					
Father	*ACVR2A*	c.16A>T	p. Lys6Ter	0 (0/133)					
Mother	*NRAS*	c.35G>A	p. Gly12Asp	0 (0/214)					
Mother	*ACVR2A*	c.A16T	p. Lys6Ter	0 (0/208)					
Infant-1	*NRAS*	c.35G>A	p. Gly12Asp		0 (0/590)				
Infant-1	*NRAS*	c.35G>A	p. Gly12Asp						0 (0/3003)
Infant-2	*ACVR2A*	c.16A>T	p. Lys6Ter			1.31 (5/376)			
Infant-2	*ACVR2A*	c.16A>T	p. Lys6Ter				0 (0/3994)		
Infant-2	*ACVR2A*	c.16A>T	p. Lys6Ter					0 (0/782)	
Infant-2	*ACVR2A*	c.16A>T	p. Lys6Ter						0 (0/1888)

Note: [a]In the parenthesis, the numerator is the number of positive reaction units for mutations, whereas the denominator is the number of total (i.e., positive and negative) reaction units for mutations. PBMC, peripheral blood mononuclear cells.

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frequently (15–87%) in HB (Lopez-Terrada et al., 2009; Purcell et al., 2011; Taniguchi et al., 2002). The majority of alterations are point mutations or deletions in exon 3 of *CTNNB1*, which results in the impairment of phosphorylation of the β-catenin protein (Rubinfeld et al., 1996). The activity of the β-catenin protein is regulated by the destruction complex composed of AXIN-1, GSK-3β, CK-1, and APC proteins, which degrade other proteins through phosphorylation and subsequent ubiquitination (Clevers, 2006; Ng et al., 2022; Taniguchi et al., 2002). Although the pathogenic germline variant (*APC*, c.3231_3237del) was found in C1 and C2 tumors, no second hit mutations were identified in the tumors. Since immunohistochemical analysis revealed accumulated β-catenin protein in the tumors, it is natural to think that the degradation of β-catenin was abrogated in the tumor cells. Thus, there may be undetectable somatic alteration(s) in the regulatory or deep intronic regions, or structural alterations, in the APC gene. Alternatively, other nongenetic events, such as epigenetic changes, could be involved in the loss of APC function in the cells.

In addition to the *APC* mutation, tumor cells harbored additional driver mutations, including the *NRAS* mutation in C1 and the *ACVR2A* mutation in C2. These mutations are not likely to be their early event, since the VAF was not high (i.e., 7.1% for *NRAS* and 14.2% for *ACVR2A*). However, tumor cells harboring these mutations might have impairments in growth and/or development. Notably, *ACVR2A* mutations are frequently identified in NASH-HCC compared to HCC caused by other etiologies (Pinyol et al., 2021). In addition, it has been reported that *ACVR2A* and *ACVR1B* mutations are frequently detected in colorectal cancer and that these genes play an important role in the carcinogenesis as tumor suppressors (Takeda et al., 2019).

To assess the clonality of each tumor, we decided to use these mutations for multiregional mutational profiling using dPCR. In contrast to the WES approach, dPCR is intended to detect extremely rare variants down to the range of 0.001% VAF, for which an NGS assay would not be able to detect mutations (Diaz & Bardelli, 2014). With the dPCR technology, the multiregional profiling revealed that both mutations were present in both tumors. Interestingly, among a total of 13 regions of tumors, the VAFs of *NRAS* were consistently higher in C1 tumor regions compared to those of the C2 tumor regions, whereas VAFs of *ACVR2A* were higher in C2 tumor regions compared to C1. However, the VAF of these mutations seemed too low to be considered a truncal mutation (Frankell et al., 2023), suggesting that these mutations may not play a central role in the development of HBs. A potential explanation for these findings is that the *APC* germline mutation has impaired degradation of β-catenin, which could drive cell division susceptibility for malignant transformation without additional somatic mutations. However, since no other paternal family member had HB with the same *APC* germline mutation, there should be nongenetic triggers for HB development in the twins.

As another index of the presence of tumor in the body, a high VAF ctDNA (i.e., 1.31% as ctDNA) for *ACVR2A* was found in the plasma of prehepatectomy Infant-2 by a dPCR assay. The *ACVR2A* mutation seemed to have disappeared after chemotherapy and remained undetectable during the postoperative period. These results further support the finding that the *ACVR2A* mutation is derived from the C2 tumor, and not from the germline. Here, it should again be noted that the *ACVR2A* mutation was found in the C1 tumor, which was physically separated from the C2 tumor circulation system. It is extremely unlikely that these tumors arose independently with the same somatic mutation. Therefore, as our multiregional dPCR assay demonstrated, it can be reasonably hypothesized that the tumor cells implanted via the maternal and fetal circulation systems during which time the tumors were not yet visible using routine detection modalities (Shatara et al., 2019). The undetectable tumor cells continued to proliferate after birth, and both tumors subsequently became detectable during infancy resulting in two independent chimeric tumors. Indeed, it has been reported that blood chimerism, which is defined as a blood cell genotype, contains different cells derived from two or more distinct zygotes in monochronic dizygotic twins, suggesting that mixed zygotes may result in microchimerism in organs (Lee et al., 2014). Although limited to monochorionic dizygotic twins, a systematic review by Peters et al. (2017) reported that chimerism was demonstrable in >90% of 31 twins they investigated. The authors suggest that, as most of the chimeric twins were discovered by accident, the phenomena may be far more common than originally speculated. In fact, bidirectional transplacental cell trafficking between mother and fetus during pregnancy has been one of the emerging topics in studying microchimerism, which is defined as an organism being made up of more than one genetically distinct individual (Boddy et al., 2015; Comitre-Mariano et al., 2022). In the present study, the microchimerism was confirmed only by tumors of the twins. The fact that both tumors acquired microchimerism consisting of somatic mutations before birth suggests that the HB development initiated during pregnancy. Moreover, the fact that the tumor cells should have been trafficking systemically in the fetus, but only arose in the liver, suggests that some tissue environmental factors may accelerate tumor progression. Although a premature alloimmune system contributing to the establishment of tumor implantation has been suggested (Arakawa et al., 2021), our findings further imply that an organ-specific cell-trafficking environment may exist during HB development.

Overall, we propose a potential order explaining the development of HBs in the monozygotic twins as follows: (1) the germline *APC* mutation gave rise to a susceptible genetic background to each tumor; (2) these tumors separately acquired different nongenetic triggers of tumorigenesis during pregnancy; (3) a small portion of each tumor traveled via maternal circulation and implanted in the liver of the counterpart (the implantation continued until birth); and (4) the triggered tumor cells kept proliferating after birth.

This study has the following limitations. Germline mutations were only accessible within two generations since the other three affected individuals (grandparents of the twins) had passed away. Due to clinical priority, only a limited amount of prechemotherapeutic biopsy specimens was available from Infant-1 (C1), whereas posthepatectomy specimens were all postchemotherapeutic tissues (C1). Specimens from Infant-2 were only posthepatectomy (C2). As a result, the quality and reproducibility for sequencing analysis might have been restricted. In fact, nongenetic alterations have not been fully assessed. Blood samples were not periodically obtained due to technical difficulties and infant conditions. Although two SNVs were used for cellular migration tracking, the mechanism of metachronous tumor development remains unknown. Finally, we were unable to examine the microchimerism in tissues other than liver, and we were not able to assess those of their mother.

In conclusion, we have identified somatic driver mutations, an *NRAS* activating mutation and a loss of function *ACVR2A* mutation, in HBs that developed in monozygotic twins with a pathogenic germline *APC* variant. The importance of these driver mutations in the development of HB remains to be elucidated.

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However, our data suggest that blood and cells of the twins could be mutually transmittable via the utero-placental maternal circulation; and HB tumorigenesis began during the fetal stage after organ development of the liver. We recommend performing periodical surveillance in twins with tumors to assess for malignancy at young ages if one of the twins exhibits a malignant tumor at that age. These findings and recommendations are based on the evidence for tumor cell microchimerism and shared tumor-development in the utero-placental environment.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/thg.2025.10019

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Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008. This study was approved by both the Iwate Medical University (HGH28-13; HG2020-028) and the University of Tokyo (27-37-1015; 2020-36-1001).

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Publication ethics. All authors have approved the final version of the manuscript and agreed to publish this work. Individual contributions are as follows: AA, SM, YF, SSN desined the study; AA, KY, KT, YA, YH, MM, HN, KI, TI collected clinical data;AA, KY, KT, ES, SI, SM, YF performed sequencing and immunohistochemistry; AA, TI, and SSN performed digital PCR; AA, YF, SSN draft manuscript; AA and SSN prepared Figures 1, 2 and 3; AA, KY, ES, SI prepared Tables 1 and 2; AA, TI, SSN prepared Supplementary Table 1; KI prepared Supplementary Figures 1 and 2; Critical discussion, AA, KY, KT, YF, SSN.

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