Altered microRNA Expression Patterns in Hepatoblastoma Patients

Abstract
Liver cancers in children are rare representing only 1.1% of malignancies, with an annual incidence rate of 1.5 cases per million. Hepatoblastoma and hepatocellular carcinomas are the most common malignancies of the liver occurring in young people aged 15 years or younger. Molecular basis of both tumors are still unclear, and common markers (i.e., CTNNB1, APC, IGF-2) are not always useful in the characterization of sporadic forms; in this respect, microRNA recently associated with carcinogenesis could play a pivotal role in their onset. CTNNB1 and APC were analyzed by sequencing, and IGF-2 promoter methylation status was assessed by methylation-specific polymerase chain reaction. MicroRNA expression was assayed by microarray and quantitative reverse transcription–polymerase chain reaction in hepatoblastoma samples. Although few genomic alterations were detected in our samples, an altered expression of some microRNA in hepatoblastoma was observed. Unsupervised clustering shows that microRNA profile can distinguish tumor from nontumor tissues. Further analyses of microRNA contents in hepatoblastoma compared with hepatocellular carcinoma highlighted four upregulated microRNA (miR-214, miR-199a, miR-150 \(P < .01\), and miR-125a \(P < .05\)) and one downregulated microRNA (miR-148a \(P < .01\)). In conclusion, although our samples were poorly informative from a genetic point of view, they showed a peculiar microRNA expression pattern compared with nontumor tissues and hepatocellular carcinoma. MicroRNA could represent valid markers for the classification of pediatric liver tumors.

Introduction
Hepatoblastoma (HB) and hepatocellular carcinoma (HCC) are two different subtypes of primary tumors arising from liver parenchymal cells [1]. Hepatoblastoma is a rare liver cancer with an incidence in western countries of 1.5 cases per million of individuals younger than 15 years. Despite this, it is the most common hepatic malignancy during childhood [1–3]. By contrast, HCC is a common solid cancer in adults: it is a complex and heterogeneous malignancy with several genomic alterations. Aberrant activation of several signaling cascades,
such as, phosphoinositide 3-kinase/mammalian target of rapamycin, Wnt, Hedgehog, insulin-like growth factor 2 (IGF-2), and apoptotic signaling have been extensively described in both HB and HCC [1,2].

Most cases of HB are sporadic, but children with familial adenomatous polyposis and Beckwith-Wiedemann syndrome have a higher risk for HB [1]. Although specific chromosome aberrations have not been linked with prognosis or casual factors, genetic disorders have been described in chromosomes 1q, 2q, 7q, 8, 17q, and 20 [4–6].

Activation of wingless (Wnt) signaling pathway through mutations in β-catenin (CTNNB1) may contribute to the development of HCC and HB [7–10]. β-Catenin is the central mediator of the well-known Wnt/β-catenin signaling pathway, which acts as a gene transcription (co)factor activating the T-cell factor/lymphoid enhancer factor [7,8].

In HB, mutations leading to constitutive activation of β-catenin have been identified in genes of the Wnt/β-catenin pathway: they occur with high frequency (48%-89%) in exon 3 of CTNNB1 and are mainly characterized by deletions or point mutations [10]. Moreover, an increased level of gene expression of IGF-2 compared with normal livers has been reported in a high proportion of human HB. In the fetal liver, promoters P2, P3, and P4 are active and expressed monoallelically; P3 is the most active promoter and P1 is inactive. However, in the adult liver, P1 becomes dominant and is biallelically expressed, and P2, P3 and P4 activities are decreased or lost [11]. In addition, P4 hypomethylation has been shown in several human cancers, including HCC, suggesting that it may be an end early common event in hepatocarcinogenesis [12]. Although the exact mechanism leading to these alterations is not entirely certain, it seems that loss of imprinting (LOI) and/or promoter demethylation are at least partly involved [13].

MicroRNA (miRs), an abundant class of noncoding small RNA ~21 nt, have been shown to play important roles in various cellular and pathogenic processes, including cellular development, immunologic response, and carcinogenesis [14–17]. Typically, they can modulate gene expression by regulating the messenger RNA at a post-transcriptional stage, aligning with sequences in the 3′-untranslated region [14,18–20]. MicroRNA can target oncogenes or tumor suppressor genes, contributing to the initiation and progression of many human cancers [21–23]. Several microRNA have been linked to initiation and progression of human cancers: miR-15a and miR-16 in chronic lymphocytic leukemia [24]; miR-145 in colon carcinoma, lung, breast, and prostate [25–29]; and miR-21 in glioblastoma [30]. Many well-known proteins involved in carcinogenesis are regulated by microRNA; for example, phosphatase and tensin homolog (PTEN) tumor suppressor is regulated by miR-21 and miR-221 [31,32], tumor suppressor protein p21waf1 is regulated by miR-221 and miR-222 [33], E2F1 transcription factor is regulated by the miR-17-92 cluster [34], and BCL-2 antiapoptotic gene is regulated by miR-15a/miR-16 cluster [22].

Despite recent studies show that many microRNA are dysregulated in HCC [29,35–40] such as miR-145, miR-199a, miR-21, miR-34a, miR-221/222, and miR-224, no data are available on microRNA expression in HB patients yet.

In this study, we analyzed samples from nine HB patients, namely, three snap-frozen and six paraffin-embedded (FFPE) tissues. Gene expression profiling for microRNA on three snap-frozen matched samples was carried out to evaluate the global content of microRNA; results were validated by quantitative reverse transcription–polymerase chain reaction (qRT-PCR) in the same samples and in six additional matched (FFPE) tissues.

### Materials and Methods

#### Tissue Samples and Clinical Data

The tissue samples analyzed in this study were obtained from patients undergoing a surgical procedure to remove a portion of the liver at the “Bambino Gesù” Hospital (Rome, Italy). Written informed consent for biologic studies was obtained from all patients analyzed, in accordance with the Declaration of Helsinki. Pathologic analysis by the institute providing the surgical specimens confirmed the histopathologic diagnosis of the samples taken for research.

We included in our study nine HB tumor samples (HBT) and their surrounding tissues not showing neoplastic features (from now called “normal tissue NT”). Their sex distribution was as follows: six males and three females (Table 1). All patients underwent SIOPEN chemotherapy treatment protocols. Six matched samples were FFPE (HBT1/NT1, HBT3/NT3, HBT5/NT5, HBT7/NT7, HBT9/NT9, and HBT11/NT11), whereas the three remaining samples were fresh-frozen (HBT13/NT13, HBT15/NT15, and HBT17/NT17). Tissues were collected from children aged between 8 and 39 months.

#### Nucleic Acid Extraction

DNA was extracted from samples using a standard proteinase K digestion and phenol/chloroform extraction; DNA was stored at +4°C in Tris-EDTA solution.

RNA was extracted using a standard Trizol RNA (Invitrogen, Milan, Italy) extraction method according to the manufacturer’s instructions. The quality of nucleic acids was assessed by agarose gel electrophoresis, and concentrations were determined through Nanodrop ND-1000 (Thermo Scientific, Waltham, CA).

#### Mutational Analyses of DNA

DNA from samples was amplified by PCR with specific primers tailored for exon 3 of CTNNB1 gene (BCTAT-1/BCTAT-2: 5′-GATTGTGAGTTGGACATGG-3′; 5′-TGTTCTTGGATGGAAG-GACTGAG-3′) and exon 15 of APC gene (Fw: 5′-ACCTCCACCA-CAAAATCACG-3′; Rev: 5′-CTTTTGAGTACTGAGGAAA-3′). After PCR amplification, products were purified using spin columns (QIAquick PCR purification kit; Qiagen, Inc., Milan Italy) and sequenced (CEQ 2000 XL DNA Analysis System; Beckman Coulter, Fullerton, CA).

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age (months)/Sex</th>
<th>Histologic Subtype*</th>
<th>CTNNB1†</th>
<th>APC‡</th>
<th>IGF-2§</th>
</tr>
</thead>
<tbody>
<tr>
<td>HB1</td>
<td>8/♂</td>
<td>Epithelial (E/F)</td>
<td>WT</td>
<td>WT</td>
<td>ND</td>
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<td>HB3</td>
<td>39/♂</td>
<td>Epithelial (E)</td>
<td>WT</td>
<td>WT</td>
<td>ND</td>
</tr>
<tr>
<td>HB5</td>
<td>17/♂</td>
<td>Epithelial (E)</td>
<td>WT</td>
<td>WT</td>
<td>ND</td>
</tr>
<tr>
<td>HB7</td>
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<td>Epithelial (E)</td>
<td>WT</td>
<td>WT</td>
<td>ND</td>
</tr>
<tr>
<td>HB9</td>
<td>24/♀</td>
<td>Mixed</td>
<td>A34D</td>
<td>WT</td>
<td>ND</td>
</tr>
<tr>
<td>HB11</td>
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<td>WT</td>
<td>WT</td>
<td>ND</td>
</tr>
<tr>
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<td>Epithelial (F)</td>
<td>T405</td>
<td>WT</td>
<td>ND</td>
</tr>
<tr>
<td>HB15</td>
<td>66/♀</td>
<td>Epithelial (E/F)</td>
<td>WT</td>
<td>WT</td>
<td>ND</td>
</tr>
<tr>
<td>HB17</td>
<td>266/♀</td>
<td>Epithelial (F)</td>
<td>WT</td>
<td>WT</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Predominant epithelial component is given in parentheses: E indicates embryonal differentiation only; F, fetal differentiation only; E/F indicates fetal and embryonal differentiation detectable.
†β-Catenin exon 3 characterization by sequencing; WT indicates wild type.
‡APC exon 15 characterization by sequencing; WT indicates wild type.
§IGF-2 promoter methylation assay by MSP; ND indicates not determined; +, methylated; −, unmethylated.

Table 1. Clinical and Pathologic Characteristics of Patients with HB.
For the detection of deletions, genomic DNA from HBT and NT tissues was amplified using the primer pairs BCAT-3/BCAT-4, BCAT-1/BCAT-6, flanking exons 2, 3, and 4 of the CTNNB1 gene:

BCAT-3, 5'-AAAATCCGCTGGACATGG-3' ;
BCAT-4, 5'-TGTCGCAAGTTCTGCATC-3' ;
BCAT-6, 5'-GGAGGAGGTCCTCGAGGAGCAG-3' [41].

**Methylation-Specific PCR (MSP)**

Methylation-specific PCR (MSP) was used to assess CpG site methylation within promoters (P) 2, 3, and 4 of the IGF-2 gene: in particular, two MSP assays (P2A and P2B) were developed to evaluate methylation in P2. A lack of CpG sites within P1 limited its evaluation by MSP. Methylation-specific PCR was performed using the CpGenome DNA modification kit (Chemicon International, Milan, Italy). Briefly, 1 μg of genomic DNA was processed according to the manufacturer’s instructions. Relative locations of the MSP assays and primers used in the assays are shown elsewhere [42].

**μParaflo MicroRNA Microarray Assay**

Microarray assay was performed on a custom array by a service provider (LC Sciences, Houston, TX). A 5-μg total RNA sample was extracted as previously described. Data were analyzed by first subtracting the background and then normalizing with a cyclic LOWESS filter (locally weighted regression). Interarray quantile normalization was performed using function "quantile.normalize" from R (www.r-project.org) package "affy" using default settings [43]. Finally, data were filtered to remove probes with constitutively low expression. Across patients, we required interquartile range for log2-transformed expression values across patients to be at least equal to 0.5 or higher and for expression to be equal to log2(1000) or higher in at least two samples. Altogether, this procedure reduced the microRNA data set from 721 to 51 microRNA.

Cluster analysis was performed using the metric Euclidean distance to compute the distance matrix for HB patients based on the expression of 51 differentially expressed microRNA. To agglomerate the patients in the hierarchical cluster, we used the Ward method. Function heatmap.2 from R package gplots was used for the graphical display of the dendrogram.

**TaqMan Real-time RT-PCR**

MicroRNA from FFPE tissues were extracted using mirVana miRNA Isolation kit (Ambion, Austin, TX). To evaluate mature microRNA in a specific manner, TaqMan MicroRNA assay kits were purchased from ABI (Foster City, CA) to detect miR-145 (catalog no. 4373133), miR-214 (catalog no. 4373085), miR-451 (catalog no. 4373209), miR-195 (catalog no. 4373105), miR-125a (catalog no. 4373149), miR-150 (catalog no. 4373127), miR-199a (catalog no. 4373272), miR-148a (catalog no. 4373130), and control RNA (RNU6B, cat. catalog no. 4373381). It is a two-step protocol requiring reverse transcription (catalog no. 4366536) with a microRNA-specific primer, followed by real-time PCR with TaqMan probes (catalog no. 4324018). The fold change of the microRNA gene in the tumor samples relative to the nontumor ones was calculated using the $2^{-\Delta\Delta CT}$ method. For details, refer to the ABI user’s bulletin “Relative Quantitation of Gene

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**Figure 1.** (A) Results of the unsupervised hierarchical clustering, in which NT tissues (blue) form a separate cluster from the HB tumors (gray). The heat map shows relative levels of microRNA expression in a green (low relative expression) to red (high relative expression) scale. (B) Volcano plot: a useful graphical that plots negative log10 $P$ values (obtained from t test) on the y-axis versus mean log2 fold change on the x-axis, comparing altered microRNA expression in HB patients showing significant alteration of a small number of specific microRNA. Black dots indicate altered microRNA in HB versus NT tissues considered significant ($P < .05$).

*Pten* transcripts were also evaluated using real-time PCR. One microgram of total RNA from each sample was reverse-transcribed using the TaqMan Reverse Transcription Reagents (Applied Biosystems). For the PCR analysis, *Gapdh* was used as an internal control: *Gapdh* primers (5′-GAA GGT GAA GGT CGG AGT C-3′ and 5′-GAA GAT GGT GAT GGG ATT TC-3′) were designed using the Primer Expression software (Applied Biosystems). The PCR primers for *Pten* (5′-CGGCGAGCATCAAATGTGTTTTCAG-3′ and 5′-AACCTGCGAGTAGAAAGGCAACTC-3′) are described elsewhere [31]. Real-time PCR was performed using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). In a total volume of 20 μl of reaction mixture, 0.1 μg of complementary DNA templates were mixed with 10 μl of SYBR GREEN PCR Master Mix (Applied Biosystems) and each pair of primers at a final concentration of 100 nM. The thermal cycling conditions were set up sequentially as follows: denaturing at 95°C for 10 minutes and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Each sample was assayed in triplicates.

**PTEN Western Blot**

Frozen tissues were weighted and homogenized in an appropriate amount of lysing Buffer A (Sigma-Aldrich, St. Louis, MO), supplemented with 0.6 U/ml of benzonase (Merck KGA, Germany) and protease inhibitor cocktails (Sigma-Aldrich) according to the manufacturers’ instructions. After protein quantification by Bradford protein assay (Bio-Rad, Germany), 25 μg of whole protein extracts were loaded onto a 10% SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Immobilon, Millipore, Billerica, MA). The blots were probed with anti-PTEN monoclonal antibody (1:1000; Cell Signaling, Beverly, MA) and with 1 μg/ml anti-β-actin monoclonal antibody (Sigma-Aldrich) overnight at 4°C. After 1 hour of incubation with either an anti-rabbit or an anti-mouse secondary antibody (1:2000; GE Healthcare, United Kingdom), the enhanced chemiluminescence detection procedure was used to visualize antibody reaction (ECL Kit; GE Healthcare).

**Results**

**β-Catenin, APC, and IGF-2 Gene Characterization in HB Tissues**

Mutations in exon 3 of *β-catenin* and in *APC* gene are frequent in HB. We performed mutational analysis to identify possible mutations on both genes in our tissue samples.

Screening of HB samples showed a point mutation in HB79 patient causing a A43D substitution and a T40S substitution; no other point mutation and/or deletion were observed in *β-catenin* exon 3 and its flanking regions. We also performed mutational analysis on *APC* gene, and no mutations were detected in exon 15 in our HB samples (Table 1).

The *Igf2* gene transcription is driven by four promoters (P1-P4) that produce distinct transcripts which vary by tissue type and developmental stage. To describe *Igf2* promoter activity, we analyzed methylation status of selected CpG islands located in its promoters. The analysis was conducted in the three frozen matched samples (HBT13/NT13, HBT15/NT15, and HBT17/NT17). No differences were detected in P2, P3, and P4 promoters’ methylation status pattern in our samples; in particular, both P2 and P3 promoters were found to be methylated in all HB samples. In contrast, P4 CpG island, within promoter 4, was hypomethylated (Table 1).

**MicroRNA Expression Profile in Human HBs**

MicroRNA-related studies on cancers are currently based on the different expression profile of microRNA in cancer versus normal tissue. Differential expression of the candidate microRNA is a possible approach to study the function of microRNA in cancer pathogenesis. To assess the putative role of microRNA in HB, we performed microarray microRNA analysis.

Three matched frozen samples were measured by microarrays in a cohort of nine matched samples of HB. Unsupervised clustering (Figure 1A), performed by using processed data from microRNA microarray analysis, shows tumor samples and adjacent nonmalignant tissues classified into two groups.

To identify the differentially expressed microRNA that cause clustering of tumor versus nontumor tissues, we calculated mean log2 ratio of the signal between HBT/NT. Levels of significance and magnitude of change in expression are represented in a volcano plot (Figure 1B); 13 differentially expressed microRNA, showing a good pattern in our samples; in particular, both P2 and P3 promoters were found to be methylated in all HB samples. In contrast, P4 CpG island, within promoter 4, was hypomethylated (Table 1).

**MicroRNA Showing Different Pattern of Expression in HB Compared with the HCC.**

<table>
<thead>
<tr>
<th>MicroRNA</th>
<th>Locus</th>
<th>Expression in HB Fold</th>
<th>Expression in HCC</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Upregulated</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsa-miR-10</td>
<td>17q21.32</td>
<td>1.67</td>
<td>Up</td>
<td>[38]</td>
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<tr>
<td>hsa-miR-10b</td>
<td>2p3.11</td>
<td>1.99</td>
<td>Up</td>
<td>[45]</td>
</tr>
<tr>
<td>hsa-miR-125a-5p</td>
<td>19q133</td>
<td>0.93</td>
<td>Down</td>
<td>[36]</td>
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<tr>
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<td>1.25</td>
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<td>1.06</td>
<td>Down</td>
<td>[35]</td>
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<td>5p31</td>
<td>1.16</td>
<td>Down</td>
<td>[38]</td>
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<td>1.71</td>
<td>Down</td>
<td>[35]</td>
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<td>1.84</td>
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<td>Down</td>
<td>[35]</td>
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<td><strong>Downregulated</strong></td>
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<td></td>
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<tr>
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<td>[35,36,40]</td>
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<td>[36]</td>
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<td>[35,39,46]</td>
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<td>[35]</td>
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<tr>
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<td>1q3133</td>
<td>0.97</td>
<td>Up</td>
<td>[40]</td>
</tr>
</tbody>
</table>

MicroRNA differentially expressed in HBT versus NT samples. MicroRNA analyzed in the study and compared with data from literature (expression in HCC) are in bold face font.

*Values are calculated as mean[log2(HBT)] - mean[log2(NT)]; ΔDown indicates downregulated in HCC versus NT tissues; NC, no change in the expression pattern; ND, no data are available in the literature; Up, upregulated in HCC versus NT tissues.
level of significance ($P < .05$), are indicated. Unsupervised clustering analysis using these 13 microRNA can discriminate tumor from non-tumor tissues (data not shown), thus indicating that these microRNA could be a valid diagnostic for HB, although the small number of samples analyzed requires further future analyses.

**Five MicroRNA Differentially Expressed in HB Showed a Different Pattern Compared with HCC**

Recent evidences published on HCC point out that specific microRNA are involved in the onset and/or progression of the disease; our results were compared with different microRNA signatures published (Table 2). We validated, by qRT-PCR, the microRNA that showed a different pattern of expression compared with the HCC in all matched samples (Table 2). Analysis of selected microRNA in HB samples (Figure 2) showed that four of them were significantly upregulated in the tumor compared with the nonmalignant tissue (miR-214, miR-199a, miR-150 [$P < .01$], and miR-125a [$P < .05$]) and one was significantly downregulated (miR-148a [$P < .01$]). In contrast, miR-106a, miR-145, miR-195, and miR-451 were not significantly dysregulated (data not shown).

**MicroRNA 214 and PTEN**

The tumor suppressor *PTEN* is a negative regulator of the phosphoinositide 3-kinase signaling, and an alteration of this signaling...
cascade has been shown to give rise to different malignancies and cancer. Recent evidences show an active contribution of miR-214 and miR-21 in controlling PTEN protein level. To verify the involvement of miR-214 and miR-21 in HB samples, we measured miR-21 and miR-214 expression levels by qRT-PCR; although miR-21 was not significantly changed in all specimens, miR-214 showed an up-regulation in the tumor versus nontumor tissues (Figure 3A). Furthermore, to assess whether miR-214 exerts its function by transcript degradation or translational inhibition, PTEN protein levels were measured and found to be decreased in HBT13/NT13, HBT15/NT15, and HBT17/NT17 samples (Figure 3B), thus showing an inverse correlation with miR-214. In contrast, Pten messenger RNA was measured in all samples, and no significant alterations were detected (data not shown).

Discussion

Research approaches to pediatric rare tumors are strongly linked to the samples’ availability, and although markers (i.e., CTNNB1, APC, IGF2) have been successfully used in the identification and classification of liver cancers, in our study, the analysis of these genes was not sufficient to characterize the differences between tumor and nontumor samples. In fact, only two mutations were identified in CTNNB1 gene, and no LOI was observed in our samples [44]. The low percentage of mutations in candidate genes and the absence of LOI observed in our study may be due to the limited number of samples, which often affects the analysis performed on rare tumors. To tackle this issue, we used microRNA as a new potential way to look for putative differences in rare cancers; actually, several reports indicate that microRNA expression profile is a feasible method for cancer classification [22].

In this study, global microRNA profiling identified differentially expressed microRNA that were commonly deregulated in HB patients. We found that 33 of 51 filtered microRNA were differentially expressed: 21 of them resulted to be upregulated and 12 were downregulated. To the best of our knowledge, this is the first report about microRNA in HB patients. Moreover, we found that several microRNA showed a completely different behavior compared with HCC. In fact, although miR-214, miR-199a, miR-150, and miR-125a were upregulated in our matched samples, they were downregulated in HCC [38]. On the contrary, miR-148a was downregulated in HB and upregulated in HCC [40]. Interestingly, miR-214 has been demonstrated to induce cell survival in ovarian cancer through targeting the 3′-untranslated region of the Pten, thus leading to the down-regulation of its protein [32]. Other reports also indicate that PTEN protein levels are regulated by miR-21 in HCC [31]. PTEN is instrumental in regulating the balance between growth and death in several cell types because it regulates Akt signaling pathway and it is misregulated in several HB cases.

To assess the role of miR-214 or miR-21 in the regulation of PTEN, we tested their expression level in our samples; although no significant change in the miR-21 expression was observed, we found miR-214 to be significantly upregulated in all our samples. Although no significant variation on the messenger level of Pten was detectable in tumors versus nontumor tissue samples, we observed a significant reduction of PTEN protein in the three matched frozen samples analyzed. We suggest that PTEN is negatively regulated by miR-214 at the protein level and that down-regulation of PTEN might correlate with elevated levels of miR-214.

Therefore, an active role of miR-214 in the activation of PTEN-mediated AKT pathway, previously described in ovarian cancer [32], could share common characteristics with other sporadic tumors; further investigations on independent cohort of samples are needed to shed light on the PTEN/AKT pathway and miR-214 in HB.

In conclusion, we pointed out for the first time that the expression pattern of 33 microRNA could represent a potential clue in HB pathogenesis, and in particular, 13 microRNA could be functional in discriminating tumor versus nontumor tissue. In addition, five microRNA (miR-214, miR-199a, miR-150, miR-125a, and miR-148a) were found to be helpful in discriminating HB from HCC, thus representing valid markers for the classification of pediatric liver tumors.

Future clarification of microRNA actions and functions will substantially improve our understanding of liver carcinogenesis. Development of drugs and molecules that specifically regulate hepatic microRNA with subsequent normalization of altered target expression may lead to novel treatments for liver cancers.

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References