Original Article
Expression of *Indian hedgehog* is negatively correlated with APC gene mutation in colorectal tumors

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**Abstract:** The regulatory mechanism of Indian hedgehog (IHH) in colorectal carcinogenesis has not been elucidated. In the current study, the expression of IHH were investigated in 7 digestive tract cancer cell lines, and in 10 normal colorectal mucosas (NCs), 30 hyperplastic polyps (HPs), 35 colorectal adenomas (ADs), and 40 colorectal adenocarcinomas (CAs) by semi-quantitative RT-PCR and immunohistochemical staining. Moreover, the mutational status of adenomatous polyposis coli (APC) and β-catenin in these tumors were analyzed by direct sequencing. IHH mRNA was lost in the 4 colon cancer cell lines harboring APC mutation. IHH mRNA was significantly decreased in CAs (0.17 ± 0.22), compared with that in ADs (0.38 ± 0.35) and HPs (0.56 ± 0.38, P < 0.05). IHH protein was expressed at a very low level or absent in both ADs (7.51 ± 11.92) and CAs (5.15 ± 9.21) in comparison to that in HPs (19.47 ± 17.91) and NCs (42.40 ± 13.67, P < 0.05). Moreover, APC mutations were negatively correlated with IHH mRNA expression (Spearman’s R = -0.636, P < 0.01) and IHH protein expression (Spearman’s R = -0.426, P < 0.01). In conclusion, down-regulation of IHH expression might be an early event during the carcinogenesis of colorectal cancer. The activation of Wnt signaling by APC mutation might contribute to the down-regulation or loss of IHH expression in colorectal tumors.

**Keywords:** Indian hedgehog, Wnt signaling pathway, colorectal cancer, APC mutation

**Introduction**

The Hedgehog (Hh) and Wnt pathways are key signaling pathways responsible for embryonic development, cellular proliferation, and adult tissue homeostasis [1, 2]. Recent studies found that these pathways may serve as the driving mediators of tumorigenicity and metastatic potential [3-6].

Ligand-dependent (i.e., ligand overexpression) activation of Hh pathway has been observed in digestive tract malignancies, including colorectal cancer [7, 8]. There are 3 vertebrate homologues of Hh ligands: Sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh). SHH has previously been shown to be overexpressed in digestive tract cancers, and contributes to the ligand-dependent activation of Hh pathway in these tumors [9, 10]. In contrast, previous studies including ours have shown that IHH may act as a competitive inhibitor of SHH, and was lost from colonic adenomas and cancers [11-13].

However, the regulatory mechanism of IHH in colon carcinogenesis has not been fully elucidated. Recently, a novel Wnt-Hh axis in colonic epithelial renewal and carcinogenesis was proposed [11, 14]. IHH is negatively regulated by Wnt signaling according to the theory. More evidence is needed to confirm this hypothesis.

In the current study, we investigated the expression of IHH in 7 digestive tract cancer cell lines, and in neoplastic colorectal tissues. We also looked for *adenomatous polyposis coli* (APC) and β-catenin mutations in these cell lines and primary tumors, in an attempt to clarify the possible regulatory mechanism of IHH expression during colorectal carcinogenesis.

**Materials and methods**

**Primary tumors and cell lines**

We collected prospectively 115 fresh samples from the patients who underwent colonoscopic biopsy or polypectomy in The Affiliated Hospital
of Luzhou Medical College for the period January 2011 to November 2012. Samples were frozen immediately in liquid nitrogen and stored at -80°C until required, whereas the other parts of samples were fixed in 10% formalin and embedded in paraffin. 30 hyperplastic polyps (HPs), 35 colorectal adenomas (ADs), 40 colorectal adenocarcinoma (CAs) (formalin-fixed, paraffin-embedded and frozen samples) were used in the study. Specimens of 10 normal colorectal mucosas (NCs) were used as controls. Clinicopathologic data for each patient were obtained from hospital records. Informed consent was obtained from all the participants. The project was approved by the institutional review board.

Seven human digestive tract cancer cell lines were also included in our study. Human colon cancer cell lines of different grade, HCT8, SW1116, SW480, and Lovo cells, esophageal cancer cell line ECA109 and gastric cancer cell lines MGC803, BGC823 were all obtained from American Type Culture Collection (Manassas, VA). Cells were grown in RPMI1640. Culture media were supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 Ag/mL streptomycin at 37°C in 5% CO₂.

RNA extraction and semi-quantitative RT-PCR

Total RNA was extracted from fresh tissue samples and cell lines using TRizol reagent (TaKaRa, China) according to the manufacturer’s instructions. 3µg of total RNA were reversely transcribed using First Strand cDNA Synthesis Kit (Fermentas, Lithuania). PCR amplification was performed in the presence of Ex Taq DNA polymerase (TaKaRa, China). Sequences of the primers used in this study are presented in Table 1. All primers used in our study were synthesized by Invitrogen. Each RT-PCR product (5 µl) was analyzed by electrophoresis through a 1.5% agarose gel. PCR amplification signals were quantified by densitometric scanning using Image-Pro Plus.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Product length</th>
</tr>
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<tbody>
<tr>
<td>IHH</td>
<td>CGGCTGACATACACGAGGC</td>
<td>GAAGCTGCCCCTTCTAGCAG</td>
<td>367 bp</td>
</tr>
<tr>
<td>APC1</td>
<td>TCCTTCATCACGAAACAGT</td>
<td>GCTGGATTGGTTCTAGGGG</td>
<td>445 bp</td>
</tr>
<tr>
<td>APC2</td>
<td>GGTGACGTGAGATCTCTTGTG</td>
<td>GATGACTTTGGGATGCA</td>
<td>823 bp</td>
</tr>
<tr>
<td>β-catenin</td>
<td>CCAATCTAATGCTAATACTG</td>
<td>CTGCATTCTAGTCCAGTAGG</td>
<td>310 bp</td>
</tr>
</tbody>
</table>

Table 1. Sequences of the primers used in the study

**Immunohistochemical analysis of IHH**

Indirect immunohistochemistry was performed with formalin-fixed, paraffin-embedded tissue sections. Antigens were retrieved by autoclave pretreatment in high pH buffer (EDTA, PH8.0) for 2 minutes at 120°C. Endogenous peroxidase activity was inhibited by incubation with 0.3% hydrogen peroxide in methanol for 10 minutes. Sections were incubated in primary antibody of IHH (1:100; C-15, sc-1196; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C over night and then washed with PBS. After the appropriate secondary antibodies, the labeled antigens were visualized by the development of brown pigment via a standard 3, 3'-diaminobenzidine protocol. Slides were then counterstained lightly with hematoxylin. Normal colonic tissues were included as positive controls. Staining without primary antibody was used as a negative control.

Immunoreactivities for IHH were estimated semiquantitatively, with reference to a previous report [15]. Staining was scored by percent of tumor cell positivity from 0 to 100 and staining intensity from 1 to 3 (1, weak; 2, moderate; and 3, strong). Multiplying product of the percentage of positive cells and the staining intensity was then divided by 3, making the score from 0 to 100.

**Mutational analysis of APC and β-catenin**

The mutation cluster region (MCR) from nucleotides 3570 through to 4800 of the APC gene was screened for sequence alteration by PCR of two overlapping fragments as described previously (APC1 and APC2, Table 1) [16]. The analyzed region covers 85% of all published somatic APC mutations [16]. For β-catenin, a genomic PCR fragment including exon 3 was amplified as described previously [17]. The PCR reactions were performed in the presence of high-fidelity Primestar DNA polymerase (HotStart version, TaKaRa, China). After denaturation at 95°C for 5 min, 30 cycles of PCR were carried out for 10 s at 98°C, 5 s at 60°C and 30-60 s at 72°C.
Indian hedgehog and APC mutation

Direct sequencing was performed using an ABI 3730 DNA Sequencer (Applied Biosystems, Foster City, CA, USA). PCR and sequence analysis of mutated samples were repeated twice to exclude PCR errors.

Statistical analysis

Data are presented as mean and standard deviation for continuous variables and as proportions for categorical variables. Data were analyzed using one-way ANOVA, followed by Bonferroni or Dunnett’s test for multiple comparisons. Differences in categorical variables were determined by the Chi-square or Fisher’s exact tests, as appropriate. Correlation statistics were analyzed using the Spearman correlation. Differences were considered significant if \( P < 0.05 \). All significance tests were two-tailed. All statistical tests were performed using SPSS software Version 13.0 (SPSS Inc., Chicago, IL, USA).

Results

**IHH expression was negatively correlated with APC mutation in digestive tract cancer cell lines**

Mutations in APC MCR were not detected in the 3 upper digestive tract cancer cells (esophageal and gastric) expressing IHH transcripts. In contrast, all of the 4 colon cancer cell lines lacking IHH transcripts harbored mutation in APC MCR (Table 2; Figure 1). β-catenin mutations were not detected in any of these cell lines.

**IHH expression was negatively correlated with APC mutation in primary colorectal adenomas and cancers**

IHH mRNA was significantly decreased in CAs (0.17 ± 0.22), compared with that in ADs (0.38 ± 0.35) and HPs (0.56 ± 0.38, \( P < 0.05 \)). IHH mRNA in ADs and HPs was also significantly lower than that in NCs (0.85 ± 0.29, \( P < 0.05 \)) (Figure 2A). Furthermore, IHH protein was expressed at a very low level or absent in both ADs (7.51 ± 11.92) and CAs (5.15 ± 9.21) in comparison to that in HPs (19.47 ± 17.91) and NCs (42.40 ± 13.67, \( P < 0.05 \)) (Figures 2B and 3).

No APC mutation was found in 10 NCs. APC mutation was found in 54.29% (19/35) of ADs and 62.50% (25/40) of CAs, significantly higher than that in HPs (40.00%, \( P < 0.05 \)). It’s worthy to note that APC mutations were negatively correlated with IHH mRNA expression level (Spearman’s \( R = -0.636, P < 0.01 \)) and IHH protein expression (Spearman’s \( R = -0.426, P < 0.01 \)).

Discussion

The role of IHH in colon carcinogenesis has not been fully determined. Our results showed that IHH expression was present in normal colon mucosa. Previous studies have shown that IHH

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**Table 2. Analysis of IHH mRNA expression and APC MCR, β-catenin mutations in digestive tract cancer cell lines**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tumor location</th>
<th>IHH mRNA expression</th>
<th>Mutation in APC MCR</th>
<th>β-catenin mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT8</td>
<td>Colon</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>SW1116</td>
<td>Colon</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>SW480</td>
<td>Colon</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Lovo</td>
<td>Colon</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>ECA-109</td>
<td>Esophageal</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>MGC-803</td>
<td>Gastric</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>BGC-823</td>
<td>Gastric</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

-, negative; +, positive.
Indian hedgehog and APC mutation

Figure 2. A. Relative expression of IHH mRNA in colorectal tissues. B. Immunostaining score for IHH in colorectal tissues. NC, normal colorectal mucosa; HP, hyperplastic polyp; AD, adenoma; CA, colorectal adenocarcinoma. Bar in middle, mean; points, individual value. *P < 0.05.

Figure 3. Representative immunohistochemical staining patterns for IHH protein in primary colorectal tissues. The expression of IHH was present in normal colorectal mucosa (A), but week in hyperplastic polyp (B). IHH was almost absent in primary colorectal adenoma (C) and adenocarcinoma (D). Original magnification, 200×.

was expressed by mature colonocyte in the adult colon and regulated their differentiation [11, 18]. Thus, it’s possible that there is a basal level of Hh pathway activity in an IHH-dependent manner in normal colon epithelium. In the present study, IHH expression (both mRNA and protein) were decreased in HPs, and almost absent in colorectal cancer cell lines and primary ADs and CAs. These results were consistent with our previous and other studies [11, 13], sug-
suggesting that down-regulation of IHH expression might be an early event during the carcinogenesis of colorectal cancer.

The regulatory mechanism of IHH in colon carcinogenesis has not been elucidated. Interestingly, the present data showed that all 4 colon cell lines lacking IHH expression harbored APC mutation. In contrast, APC mutation was not detected in 3 upper digestive tract cell lines expressing IHH mRNA. Furthermore, IHH expression was negatively correlated with APC mutation in primary colorectal adenomas and cancers. These results suggested that the down-regulation or loss of IHH expression in colonic tumors could be a consequence of constitutive activation of Wnt signaling.

Activation of the Wnt pathway, occurring always through mutation of APC or β-catenin, is shown to be key early events involved in the multi-step process of colorectal tumorigenesis [19, 20]. Recent studies have indicated the existence of cross-talk between the Wnt and Hh signaling pathways [11, 14, 21, 22]. IHH has been shown to restrict the activity of Wnt signaling to the base of the colonic crypt and thus maintain the differentiation of superficial epithelial cells [11]. IHH expression, in turn, is downregulated in response to Wnt signaling [11].

Indeed, a previous study reported that potential β-catenin/TCF-complex binding sites were present within the Ihh promoter in mouse [23]. On the other hand, a recent report demonstrated that IHH could repress Wnt signaling in colon cancer cells [11]. Thus, we proposed that upregulation of IHH could offer a novel target for colorectal cancer therapy by blocking Wnt signaling pathway.

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Disclosure of conflict of interest

None.

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Indian hedgehog and APC mutation


