Heparin/Heparan Sulfate Interacting Protein Gene Expression Is Up-regulated in Human Colorectal Carcinoma and Correlated with Differentiation Status and Metastasis\(^1\)

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**ABSTRACT**

We applied a subtractive hybridization strategy to obtain genes that are differentially expressed in colorectal carcinoma. Heparin/heparan sulfate interacting protein (HIP) was shown to be up-regulated in colorectal carcinoma. A study of 53 patients with documented colorectal carcinoma showed that 70% of the tumors had HIP tumor-to-normal ratios (expression in tumor tissue compared to expression in normal mucosa) of >2. In six patients with concomitant polyps, HIP expression in the polyps was similar to the carcinoma, showing that up-regulation of HIP may be an early event in tumorigenesis. A significant inverse correlation between HIP levels and the presence of distant metastasis (Duke’s stage D) was noted. Similarly, HIP expression was also related to differentiation status in human colorectal carcinoma cell lines. HIP expression was lower in the poorly differentiated COLO 205 cell line compared to the well-differentiated HT-29 cell line. The correlation was further strengthened by studies in COLO 205 cells that were induced to differentiate with herbimycin A treatment. HIP expression was significantly higher when the cells were induced to differentiate. Withdrawal of herbimycin A resulted in a reversal of morphological changes associated with differentiation and an associated decrease in HIP expression. These studies indicate that HIP is an important molecule for cell-cell and cell-extracellular matrix adhesion. The up-regulation of HIP may be an early event in tumorigenesis, and its increased expression may facilitate growth and local invasion. A lower expression of HIP in tumors results in decreased cell adhesion, favoring metastasis. HIP is a candidate marker of abnormal cell growth in the colon and a prognostic marker for colorectal carcinoma.

**INTRODUCTION**

Recently, there has been interest in characterizing cancer cells at the gene product level to determine how gene expression changes with progression of the cancer (1). The characterization of tumor cells at the phenotypic level and the correlation of phenotypic changes and tumor progression will, hopefully, shed light on the mechanisms of tumor development and provide useful genetic markers for diagnosis and prognosis. Colorectal tumorigenesis is a multistep process involving activation of oncogenes and/or the suppression of tumor suppressor genes (2–7). These, in turn, give rise to changes in gene expression and cell phenotype. The gene expression profiles in normal and gastrointestinal cancer cells were recently examined (8). However, no attempt was made to study the differential expression of individual genes or to correlate gene expression with the characteristics and behavior of the cancers. Several other differential gene expression approaches have been used and applied to the study of colorectal cancer (9–11). However, these methods often require several rounds of hybridization and are not suitable for the identification of less abundant transcripts.

In this paper, we describe the identification and characterization of differentially expressed genes in colorectal cancer using a newer subtractive hybridization strategy (12). This method has two distinct advantages over previous methods: it has a high subtraction efficiency and an equalized representation of differentially expressed sequences. The candidate genes identified were subjected to an additional differential screening process (13, 14) and then confirmed by Northern blot analysis. The expression of selected genes was then studied in patients and cell lines in an attempt to correlate gene expression with tumor behavior.

**MATERIALS AND METHODS**

**Tissue Samples.** Human colon tissue specimens were obtained immediately after surgical resection of the colon. Samples were obtained from the tumor, polyp (when present), and normal mucosa adjacent to the margin of resection. Necrotic parts of the tumors were removed. Normal colonic mucosa was dissociated from muscle and connective tissue. The samples were frozen in liquid nitrogen and transferred to a −80°C freezer for storage. The remaining resected colon with the rest of the tumor was sent for histopathological analysis.

**Cell Lines.** Human colorectal carcinoma cell lines HT-29, HCT 116, and COLO 205 were purchased from American Type Culture Collection (Manassas, VA). HT-29 and HCT 116 were grown in McCoy’s 5 A (Sigma Chemical Co., St. Louis, MO), whereas COLO 205 cells were grown in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD). Both media were supplemented with 10% fetal bovine serum (Life Technologies, Inc.). The cells were maintained in an incubator at 37°C in a 5% CO2 humidified atmosphere.

**Total RNA Extraction and Poly(A) RNA Purification.** Total RNA was prepared from the tissues and cell lines using the guanidium thiocyanate method as described previously (15). Total RNA was enriched for poly(A) RNA using prepacked oligo(dT)-cellulose spin columns (Pharmacia, Uppsala, Sweden).

**Subtractive Hybridization.** Poly(A) RNA obtained from a colon cancer and mucosa around the margin of resection was used for subtractive hybridization according to the protocol provided (PCR-select cDNA subtraction kit; Clontech, Palo Alto, CA). Briefly, 2 μg of each type of mRNA were reverse transcribed at 42°C for 1 h using Moloney murine leukemia virus reverse transcriptase and a cDNA synthesis primer provided in the kit. The cDNAs from both tissues were separately digested with RsaI to yield shorter, blunt-ended fragments. The population of cDNA fragments from tumor tissue was divided into two portions. Each portion was ligated to a different adaptor. Two rounds of subtractive hybridizations were performed. The entire population of molecules was subjected to suppression PCR using the primers and PCR parameters recommended. A secondary PCR amplification was then performed using nested primers to enrich for differentially expressed sequences. The amplified fragments were cloned into a pT-Adv vector (Clontech). The ligation products were transformed into TOPO10\(^{\text{E}}\) Escherichia coli competent cells. One hundred clones were selected for further characterization.

**Differential Screening.** The 100 clones were subjected to a differential screening process (Clontech). Briefly, 5 μl of amplified product of each candidate gene were mixed with 5 μl of 0.6 M NaOH, spotted onto duplicate nylon membranes (Qiagen; Qiagen, Chatsworth, CA) and cross-linked (Stratalinker; Stratagene, La Jolla, CA). The duplicate membranes were hybridized to \(^{32}\)P-labeled probes synthesized as first strand cDNA from the tester (colon cancer) and driver (normal colonic mucosa), respectively. The clones...
that gave at least 5-fold higher hybridization signals with the tester probe were selected for further analysis.

**Northern Blot Analysis.** Northern blot analyses were used to confirm differential expression of selected clones after differential screening. Fifteen \( \mu g \) of RNA were separated by formaldehyde/agarose gel electrophoresis, transferred to nylon membrane (Qiabran, cross-linked (Stratallinker), and hybridized to cDNA probes, as described previously (16). The cDNA fragments of selected clones were obtained by PCR and gel purification (Qiaquick; Qiagen). Relative mRNA levels were quantified by scanning densitometrym (Bio-1D Version 5.08; Vilber Lourmat, Marne-La-Vallée, France) and normalized against actin mRNA levels. T/N \( ^a \) ratios were obtained by dividing the normalized relative density units of the tumor by normal mucosa. Minor variations in gel loading were corrected by hybridization to a human actin cDNA probe after the blots were stripped. The blots were stripped by shaking in 0.5% SDS for 10 min, followed by a 5-min wash in 2\( \times \) SSC after hybridization to the cDNA probes.

**DNA Sequencing.** The clones that were confirmed to be differentially expressed in colon cancer in Northern blots were sequenced using the SequiTherm Excel DNA sequencing system (Epicentre Technologies, Madison, WI). The reaction products were separated by electrophoresis in 6% polyacrylamide gels (Stratagene). Gels were dried and exposed to Biomax MR autoradiographic films (Kodak, Rochester, NY).

**Expression of HIP and rp L7a in Human Colorectal Cancer Patients and Cell Lines.** Partial cDNAs encoding HIP (bases 476–606; Ref. 17) and rp L7a (bases 739–835; Ref. 18) were used for Northern blot analysis. The expression of the two genes was studied in a total of 53 colorectal cancer samples and normal mucosa as well as three human colorectal carcinoma cell lines, HT-29, HCT 116, and COLO 205.

**Regulation of HIP Expression in Herbimycin A-treated COLO 205 Cell Line.** COLO 205 cells were plated at a density of 1.5 \( \times 10^6 \) cells/flask (75 cm\(^2\); Falcon) and cultured for 24 h before herbimycin A (Life Technologies, Inc.; 300 ng/ml final concentration) was added to the culture medium. The medium was replaced with RPMI 1640 after 96 h of treatment. The medium was changed every 48 h during the experimental period. The morphology of the cells was examined under a confocal microscope (Zeiss, Oberkochen, Germany) at 0, 24, 48, and 96 h after herbimycin A treatment as well as 24, 72, and 120 h after withdrawal of herbimycin A. Total RNA was also prepared from the cells at the respective time points from six identical flasks. Partial cDNAs encoding HIP and villin (bases 2176–2624; Ref. 19) were used for Northern blot analysis.

**Statistical Analysis.** The mean values of different groups were compared using the one-way ANOVA. Differences in means between two groups were compared using the Student’s \( t \) test (SPSS for Windows Version 8; SPSS Inc., Chicago, IL). \( P < 0.05 \) was considered significant.

**RESULTS**

**Histopathology Report on Specimen Used for Subtraction.** The tumor specimen used for subtractive hybridization was a moderately differentiated adenocarcinoma that involved the full thickness of the colonic wall. It had spread to the regional lymph nodes, and perineural and vascular invasion were noted. The tumor was classified as Duke’s C stage. Histologically, the margin of resection was free of tumor cells.

**Identification of Genes Up-Regulated in Colon Cancer by Northern Blot Analyses.** Of the 100 clones picked, 67 clones were shown to be up-regulated at least 5-fold by differential screening. Of the 67 clones, only 20 of them had T/N ratios of \( > 2 \) in Northern blot analyses. The sequences of the 20 clones were compared to sequences deposited in GenBank using the BLASTN program (20). Eight sequences were novel, and the remaining 12 were known genes. The identities of the 20 genes are summarized in Table 1, together with the T/N ratio for each gene.

<table>
<thead>
<tr>
<th>Clone no.</th>
<th>Identity*</th>
<th>T/N ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Novel (1 kb)</td>
<td>3.24</td>
</tr>
<tr>
<td>2</td>
<td>Heparin binding protein (HIP)</td>
<td>4.60</td>
</tr>
<tr>
<td>3</td>
<td>Novel (5.5 kb)</td>
<td>2.10</td>
</tr>
<tr>
<td>4</td>
<td>Novel (1.5 kb)</td>
<td>9.76</td>
</tr>
<tr>
<td>5</td>
<td>rp L7a</td>
<td>4.40</td>
</tr>
<tr>
<td>6</td>
<td>Mitochondrial phosphate carrier protein</td>
<td>3.47</td>
</tr>
<tr>
<td>7</td>
<td>Human chaperonin</td>
<td>2.23</td>
</tr>
<tr>
<td>8</td>
<td>Alu 2 region fusion gene</td>
<td>6.25</td>
</tr>
<tr>
<td>9</td>
<td>Human Y316 gene</td>
<td>3.44</td>
</tr>
<tr>
<td>10</td>
<td>HnRNP A2 protein</td>
<td>4.01</td>
</tr>
<tr>
<td>11</td>
<td>Novel (1 kb)</td>
<td>2.15</td>
</tr>
<tr>
<td>12</td>
<td>Human lung surfactant protein D</td>
<td>2.30</td>
</tr>
<tr>
<td>13</td>
<td>Integrin linked kinase</td>
<td>4.33</td>
</tr>
<tr>
<td>14</td>
<td>Novel (0.8 kb)</td>
<td>5.21</td>
</tr>
<tr>
<td>15</td>
<td>Novel (1.5 kb)</td>
<td>9.86</td>
</tr>
<tr>
<td>16</td>
<td>Human I ( \alpha ) B related protein</td>
<td>3.25</td>
</tr>
<tr>
<td>17</td>
<td>Epithelial specific transcription factor</td>
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<td>18</td>
<td>Novel (1 kb)</td>
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<tr>
<td>19</td>
<td>hP1B secretory protein</td>
<td>3.38</td>
</tr>
<tr>
<td>20</td>
<td>Novel (2.5 kb)</td>
<td>8.11</td>
</tr>
</tbody>
</table>

* Estimated mRNA sizes of the novel genes are indicated in parentheses.

**Differential Expression of HIP in Tumors and Correlation with Metastasis.** Fifty-three patients with documented colorectal cancers were used for this study. The differentiation status, Duke’s staging, and T/N ratios of these tumors are summarized in Table 2. Thirty-seven of the 53 tumors studied (70%) had HIP T/N ratios of \( > 2 \). A significant inverse correlation between HIP levels and presence of metastasis was noted. The mean T/N ratio for tumors with metastasis was 1.45 ± 0.25 \( (n = 7) \) compared to 2.81 ± 0.19 \( (n = 46) \) for tumors without metastasis \( (P < 0.01; \text{Fig. 1}) \). Similarly, Duke’s D tumors had significantly lower HIP levels compared to Duke’s A, B, and C tumors. The mean HIP levels in the Duke’s A, B, and C groups were not significantly different from each other (Fig. 2).

**Expression of HIP in Polyps.** Six patients with colon carcinoma also had concomitant polyps in the resected colon. HIP expression in the polyps was determined in comparison with normal and colon carcinoma samples obtained from the same patient by Northern blot analysis. As shown in Fig. 3A, HIP expression was up-regulated in both polyps and colon carcinoma compared to normal mucosa. The level of HIP expression in polyps was similar to the level of expression in colon carcinoma (Fig. 3B).

**Expression of HIP and rp L7a in Human Colorectal Cancer Cell Lines.** Most of tumors in this study were moderately differentiated and did not allow a meaningful study of the possible correlation between the HIP expression and differentiation status. Hence, we used human colon cancer cell lines. HIP mRNA was easily detected in the HT-29, HCT 116, and COLO 205 cell lines. HIP expression was highest in the well-differentiated HT-29 cell line compared to the moderately and poorly differentiated HCT 116 and COLO 205 cell lines, respectively. In contrast, the expression of rp L7a, another gene that is up-regulated in colon cancer cells, was similar in HT-29 and COLO 205 cell lines and lowest in the HCT 116 cell line, showing a lack of correlation between expression of rp L7a and differentiation status (Fig. 4).

To further investigate the association between HIP expression and differentiation status, the effect of herbimycin A on HIP expression in COLO 205 cells was studied. Herbimycin A, a tyrosine kinase inhibitor, causes the COLO 205 cells to differentiate (21). In the experiment, cells were treated with herbimycin A for 96 h to induce differentiation, after which herbimycin A was withdrawn, and the
Table 2. HIP expression in colorectal cancer patients

<table>
<thead>
<tr>
<th>Duke's stage</th>
<th>No. of patients with T/N &gt; 2/no. of patients analyzed</th>
<th>Differentiation&lt;sup&gt;a&lt;/sup&gt;</th>
<th>T/N ratio&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>3/3</td>
<td>Well 0</td>
<td>2.44 ± 0.24</td>
</tr>
<tr>
<td>B</td>
<td>15/21</td>
<td>Moderately differentiated 3</td>
<td>2.68 ± 0.26</td>
</tr>
<tr>
<td>C</td>
<td>16/22</td>
<td>Poor 22</td>
<td>2.97 ± 0.31</td>
</tr>
<tr>
<td>D</td>
<td>3/7</td>
<td>Poor 7</td>
<td>1.45 ± 0.25</td>
</tr>
</tbody>
</table>

<sup>a</sup> Well, well differentiated; Mod, moderately differentiated; Poor, poorly differentiated.

<sup>b</sup> The T/N ratio is the ratio of HIP gene expression in tumor against normal tissue after correction for minor loading differences with actin.

A total of 53 patients with colorectal carcinoma were studied. The ratios of patients with T/N ratios > 2, differentiation status of the tumors, and mean T/N ratio were determined for each Duke’s stage category.

cells grown in normal RPMI 1640 for another 120 h. The effects of herbimycin A treatment and withdrawal on the morphology of the cells are shown in Fig. 5A. At 24 h after herbimycin A treatment, the cells appeared larger and flattened, were less rounded, and attached to one another to form aggregates and epithelioid colonies, which became progressively larger from 24 to 96 h of treatment. The removal of herbimycin A from the medium reversed the morphological changes by 72 h. The cells returned to their pretreatment morphology, with the cells appearing smaller, more rounded, and were less adherent to one another. By 120 h after removal of herbimycin A, the cells were indistinguishable from control cells without treatment. Differentiation of COLO 205 cells was also associated with an increase in the expression of villin (Fig. 5B), a marker of enterocyte differentiation (22). However, expression of villin had not returned to control levels 120 h after removal of herbimycin A. At this time, the morphological changes of differentiation induced by herbimycin A had completely reversed. HIP was up-regulated in herbimycin A-treated cells. A significant increase in HIP expression was evident within 24 h of treatment. Expression continued to increase with sustained treatment. HIP expression decreased to control levels within 72 h after withdrawal of herbimycin A (Fig. 6).

DISCUSSION

The identification and characterization of genes up-regulated in colorectal carcinoma help us to better understand mechanisms of tumorigenesis and metastasis. In addition, these genes may lead to the development of diagnostic and prognostic markers that may be used in patient management. The subtractive hybridization strategy applied in this study resulted in the identification of 20 genes that were shown to be consistently up-regulated in colorectal carcinomas. Among the 20 genes, one was selected for further characterization.

HIP is a cell surface binding protein originally isolated from a human uterine epithelial cell line (RL95). HIP is highly expressed in most human epithelial cell lines, including uterine and fibroblastic cells (17). It binds directly to heparin (23) and functions as a cell-cell and cell-extracellular matrix adhesion molecule (24–27). Recent studies suggested that HIP may play a role in cell migration and metastasis of melanoma and breast cancer cells (28–30). Consistent with the hypothesis that HIP can facilitate cell invasion, Rohde et al. (31) showed that HIP can assist trophoblast cells to attach to the uterine epithelium and facilitates cell invasion. To understand the significance of the up-regulation of HIP in colon carcinoma cells, we studied the expression of HIP in colorectal carcinoma samples and cell lines. Of the 53 colorectal carcinoma samples studied, 37 of them (70%) showed an increase in HIP expression of ≥2-fold compared to normal mucosa. In the six cases in which polyps were present concomitantly with the carcinoma, HIP expression in the polyps was also up-regulated. This suggests that up-regulation of HIP may be an early event in colorectal carcinogenesis. Up-regulation of HIP was also recently shown to occur in human thyroid carcinomas (32). The up-regulation of HIP in tumors would facilitate cell-cell and cell-extracellular matrix interactions that may be important for growth and local invasion of the tumor.

Interestingly, there was a significant inverse correlation between...
expression of HIP and metastasis. Tumors with metastasis (Duke’s stage D) had significantly lower levels of HIP compared to tumors in Duke’s stage A, B, and C. A higher percentage of patients in Duke’s A, B, and C stages had T/N ratios of \( \geq 2 \) compared to Duke’s stage D. The lower levels of HIP expression in tumors with metastasis is consistent with the proposed function of HIP in cell adhesion. The lower the expression of HIP, the greater the likelihood that the cells detach from the primary tumor and metastasize. It is unclear whether the lower expression of HIP in tumors with metastasis is the result of a down-regulation of HIP prior to metastasis or an intrinsic property of the tumor itself. It is possible that the tumor may down-regulate HIP expression after growing to a certain size to facilitate metastatic spread. Alternatively, it is possible that HIP expression is an intrinsic property of a tumor such that the lower the expression the greater the likelihood of metastasis.

Metastasis of tumors occurs by a complex process involving invasion, embolization, survival in the circulatory system, arrest, extravasation, and proliferation in the target organ (33, 34). The up-regulation of HIP in polyps suggest that HIP may function early in the tumorigenesis to promote growth, proliferation, and local invasion into the surrounding tissue. Subsequently, either with the passage of time or growth constraints, HIP may be down-regulated to facilitate metastasis. Alternatively, it is possible that HIP expression is an intrinsic property of a tumor such that the lower the expression the greater the likelihood of metastasis.

To understand significance of the down-regulation of HIP in tumors with metastasis, we studied the expression of HIP in cell lines. The expression of HIP was examined in HT-29, HCT 116, and COLO 205 cell lines. HT-29 is a well-differentiated cell line, whereas HCT 116 and COLO 205 are moderately and poorly differentiated, respectively. HIP expression appeared to correlate with the differentiation status of these cell lines, with the highest expression in HT-29 and lowest in COLO 205. The correlation between HIP expression and differentiation status was further strengthened by the herbimycin A studies in COLO 205 cells. COLO 205 cells are poorly differentiated. However, in the presence of a tyrosine kinase inhibitor, herbimycin A, the cells are induced to differentiate (21). The cells undergo morphological changes that are characteristic of differentiated cells. The cells differentiate to become bigger in size, are more adherent to one another and form aggregates and epithelioid colonies in the presence of herbimycin A. Villin, an enterocyte differentiation marker (22, 35–37) was up-regulated after herbimycin A treatment. However, withdrawal of herbimycin A did not decrease villin expression to control levels even after 120 h. The up-regulation of HIP associated with differentiation is consistent with the proposed role of HIP as a cell-cell and cell-extracellular matrix molecule that facilitates adhesion of the cell to one another and to the surrounding. The up-regulation of HIP would allow cells to adhere to one another, hence promoting the formation of cell aggregates. In addition, HIP would also promote adhesion to the wall of the flask, allowing the cells to spread out more and appear larger. The effect of herbimycin A on the cells is reversible. Withdrawal of herbimycin A from the medium results in the reversion of the cells to a pretreatment morphology. These changes were obvious within 72 h after withdrawal. The cells became smaller, more rounded, and less adherent and formed less aggregates. By 120 h after withdrawal, the cells were indistinguishable from control untreated cells. As
associated with the reversion of morphology, HIP expression was also decreased. This underscores the important correlation between cell differentiation and HIP expression and shows that HIP expression may be a better marker of enterocyte differentiation than villin.

The association between differentiation status and HIP expression may, in part, explain the association between HIP expression and the occurrence of metastasis in patients. It is well known that poorly differentiated cells generally exhibit more malignant behavior. In colorectal carcinoma cells, a characteristic of poorly differentiated...
cells is decreased cell adhesion. The decreased cell adhesion, which may be explained by a decrease in HIP expression, would favor metastasis of the cells to distant sites.

In summary, we have shown that HIP is up-regulated in disordered growth of the colon. Both polyps and colorectal carcinoma have higher expression of HIP compared to normal mucosa derived from the same patient. The up-regulation of HIP may be an early event in tumorigenesis of colorectal carcinoma. Interestingly, lower HIP expression is associated with presence of metastasis and a poorly differentiated status of the cells. This is consistent with the function of HIP as a cell adhesion molecule. The correlation of HIP expression with tumorigenesis, differentiation status, and tumor behavior may allow HIP to be developed as a marker for abnormal cell proliferation with tumorigenesis, differentiation status, and tumor behavior.

REFERENCES

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