Granulin–Epithelin Precursor Overexpression Promotes Growth and Invasion of Hepatocellular Carcinoma

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ABSTRACT

Purpose: Granulin–epithelin precursor (GEP) is a novel growth factor. Our earlier cDNA microarray study indicated that GEP was overexpressed in hepatocellular carcinoma (HCC). The aim of this study was to investigate the clinical significance of GEP expression and its potential as a therapeutic target in HCC.

Experimental Design: A total of 110 pairs of HCCs and adjacent non-tumor liver tissues, and 22 normal liver tissues were examined. The GEP RNA level was examined by quantitative reverse transcription-PCR, and protein localization by immunohistochemistry. The GEP function was examined by transfection experiments.

Results: The RNA levels of the HCCs were significantly higher than those of the nontumor liver tissues and normal livers (P < 0.001). GEP protein staining was observed in tumor cytoplasm, and the GEP protein levels of the HCCs were also significantly higher than those of the nontumor liver tissues and normal livers (P < 0.001). The majority of HCCs demonstrated up-regulation of GEP protein compared with their adjacent liver tissues [79 (71.8%) of 110]. Positive correlation of GEP RNA with protein levels was observed in HCCs (P < 0.01). Strong GEP expression was associated with large HCCs, venous infiltration, and early intrahepatic recurrence (P < 0.05). Functional studies on the HCC cell line Hep3B demonstrated that reduction of GEP protein levels resulted in decreased cell proliferation rates, tumor invasion ability, anchorage-independent growth in soft agar, and tumorigenicity in nude mice (P < 0.05).

Conclusion: GEP is an important factor for HCC growth, invasion, and metastasis. GEP has the potential to serve as a tumor marker and therapeutic target.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide, with about a half million new cases and almost as many deaths per year (1–3). Better understanding of the etiological factors and molecular basis of the disease is crucial to disease prevention and management. Epidemiologic studies have shown that hepatitis B and C virus infections, alcohol-induced liver injury, and consumption of aflatoxin are closely associated with HCC. However, little is known about the molecular basis of liver cancer development and progression. The p53 tumor suppressor gene is believed to play a major role as a “cellular gatekeeper”, whereas β-catenin oncogene deregulation has recently demonstrated a neoplastic transformation potential (2–4). However, the major growth factor in liver carcinogenesis is largely unknown.

Using the cDNA microarray approach, we have identified 1,648 differentially expressed genes in HCCs and adjacent nontumor liver tissues (5). About one half of these genes showed a significantly higher expression level in HCCs and one half of them a significantly lower expression level in HCCs. Attention was drawn to genes that demonstrated higher expression levels in tumors [759 (46%) of 1,648] with the aim to search for potential tumor markers. Among the highly expressed genes in HCC, we focused on the granulin–epithelin precursor (GEP) with gene locus at 17q21.32, the region frequently reported with chromosome gain in liver cancers (6, 7), strongly suggesting the presence of important growth factor(s) proto-oncogene(s). GEP is a novel growth factor and a secretory protein that is capable of stimulating cell proliferation (8). Its reduced expression is associated with inhibition of tumorigenic potential (9–10). No studies have reported the GEP expression pattern and its biological role in HCC. In this study, we examined the RNA level and protein localization of GEP in HCCs, their corresponding nontumor liver tissues, and normal liver tissues. We also examined the function of GEP by transfection experiments.

PATIENTS AND METHODS

Patients and Specimens. Between March 1999 and May 2002, 110 patients undergoing resection of HCC at Queen Mary Hospital, the University of Hong Kong were recruited in the present study. HCC and adjacent nontumor liver tissue specimens were collected after informed consent had been obtained from the patients. The study protocol was approved by the Ethics Committee of the University of Hong Kong. The first 55
HCCs were examined under a cDNA microarray study. The second 55 HCCs were used for validation of the observations in the microarray study by quantitative reverse transcription-PCR (RT-PCR). All of the 110 HCCs were studied by immunohistochemistry for detection of GEP protein. The age of the patients ranged from 13 to 79 years, with a median age of 52 years. There were 89 men and 21 women. Serum hepatitis B surface antigen was positive in 100 patients (90.9%). Tumors were staged according to the pathological tumor-node-metastasis (pTNM) staging system, 1997 version (11). The latest 2002 version was not used because it did not clearly stratify patients with advanced tumor stages according to the survival rate (12). Distribution of the pTNM stages and other clinicopathological features is listed in Tables 1 and 2. The patients were closely monitored for recurrence by computed tomography. Thirty-two of 110 patients had intrahepatic recurrence in the first year.

Normal liver specimens from 22 organ donors were collected during transplantation operations performed at the same institution from May 2000 to October 2002. The organ donors had no underlying liver diseases and were negative in the hepatitis B serology test.

Each tissue specimen, 0.5 to 1 cm³, was divided into three equal portions. One portion was formalin fixed and paraffin embedded for histological and immunohistochemical studies. The other two portions were snap-frozen in liquid nitrogen and stored at −70°C until use. Total RNA was extracted with the RNeasy kit (Qiagen, Hilden, Germany).

Quantitative Reverse Transcription-PCR. Quantitative RT-PCR was performed as described previously (13), with human 18s rRNA as the normalization control for subsequent multiplexed reactions (14). Quantification was performed with the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA). Primers and probes for GEP were GRN-forward (5’-CAA ATG GCC CAC AAC ACT GA-3’) and GRN-reverse (5’-CCC TGA GAC GGT AAA GAT GCA-3’), and GRN-probe (5’-6FAM CCA CTG TCT CCG CCA CTC MGBNFQ-3’). Primer and probe reagents for control H11032) (Invitrogen, Carlsbad, CA). The full-length GEP was subcloned with the NorI and XbaI restriction sites. The NH2-terminal fragment (size of 290 bp, corresponding to position −31 to 258bp; refs. 9–10) was generated by PCR, and subcloned in antisense and sense orientation to generate the respective constructs. The human HCC cell line Hep3B (American Tissue Culture Collection, Manassas, VA) was used for functional studies. The cells were maintained under standard culture condition with serum-containing DMEM (supplemented with 10% FBS, 50 units/mL Penicillin G, and 50 μg/mL streptomycin). The cells were transfected with Lipofectamine (Invitrogen) according to the manufacturer’s instruction. Four sets of transfection experiments were performed: (1) antisense fragment to decrease the GEP level; (2) full-length for overexpression of GEP; (3) sense fragment as control for antisense experiment; and (4) empty vector as control for all of the transfection experiments. Stable clones were selected by G418. The GEP protein level and proliferation were assessed in serum-containing (10% serum) and serum-starved (0% serum) conditions.

Proliferation Assay. Cell proliferation was assayed by seeding 50,000 cells into 6-well plates. Cells were harvested every day for 5 consecutive days, and viable cells were counted by trypan blue exclusion. Cell activity was measured via mitochondrial dehydrogenase activity performed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (16–17), in which 5,000 cells were seeded into 96-well plates and assayed for 5 consecutive days.

Invasion Assay. The cell invasion ability was determined with the BioCoat Matrigel invasion chamber (BD Biosciences, Bedford, MA), in which the chamber membrane filter (8 μm pore size) was coated with the BD Matrigel Basement Membrane Matrix (BD Biosciences). The upper chamber was loaded with 50,000 cells in 2 mL of serum-free medium, and the lower chamber was filled with 2 mL of serum-containing medium. After 48 hours of standard incubation, noninvading cells on the upper surface of the membrane were removed with cotton swabs. Invading cells on the lower surface of the membrane...
were washed in PBS, fixed in Carnoy’s solution, and stained with hematoxylin and eosin. The invading cells were counted under the microscope in 10 randomly selected fields for each membrane filter (×100).

**Colony-Formation Assay.** Anchorage-independent growth was assessed by colony-formation ability in soft agar (18). The agar base of 1.5 mL in a 6-well plate was formed by mixing an equal volume of 1.6% low-melting agar (USB) and 2× DMEM supplemented with 20% fetal bovine serum. The 5,000 cells were suspended in 1.5 mL of soft agar (mixture containing 2× DMEM supplemented with 20% fetal bovine serum and 0.8% low-melting agar) and were overlaid on the agar base. After 4 weeks, colonies over of 15 cells were counted under the microscope in 10 fields per well. Each data point for in vitro experiments represented results from at least three independent experiments performed in duplicates.

**Tumorigenicity Assay.** BALB/c athymic nude mice of 4 weeks old were used to test the in vivo tumorigenicity potential of the transfectants (19). The study protocol was approved by the Committee on the Use of Live Animals in Teaching and Research at the University of Hong Kong. Five million cells were inoculated subcutaneously at the dorsal region of the trunk of each animal. The tumor size and body weight were measured twice weekly. The mice were killed on day 60 with the tumor harvested for further examination. Each of the experimental groups consisted of 5 mice.

**Statistical Analysis.** Preoperative clinical parameters, pathological data of the resected specimens, and follow-up data were prospectively collected and recorded in a computerized database. Continuous data were presented as median value (interquartile range). Replicate experimental results were presented as mean values (SD). Statistical analysis was carried out with a statistical software (SPSS version 9.0 for Windows; SPSS Inc., Chicago, IL). The comparison of categorical variables was examined by χ² test, or Fisher’s exact test for small sample size. Continuous variables were assessed by Spearman correlation, and compared between groups by the Student’s t test. Difference was considered statistically significant if the P value was less than 0.05.

**RESULTS**

**Granulin–Epithelin Precursor RNA Level.** The earlier observation that GEP was overexpressed was based on the cDNA microarray study on the first 55 HCCs and their adjacent nontumor liver tissues (5). To test the robustness of the microarray research method, we randomly examined 39 (from the 55) HCC pairs in the first sample set and used quantitative RT-PCR to examine the GEP transcript levels. By quantitative RT-PCR, the GEP expression levels in HCCs (median, 6.3; interquartile range, 5.4–7.2) were also significantly higher than those of the adjacent nontumor liver tissues (median, 4.4; interquartile range, 3.9–5.1; P < 0.001). The GEP levels examined by the two research methods showed a high concordance in the HCCs (r = 0.643, P < 0.001; Fig. 1A).

To validate the observation, we recruited an independent sample set (the second 55 pairs of HCCs and nontumor liver tissues) and used real-time quantitative RT-PCR to measure the transcript levels (Fig. 1B). The results indicated that the HCCs (median, 6.1; interquartile range, 5.2–7.0) demonstrated a significantly higher GEP transcript level than did their associated nontumor liver tissues (median, 4.4; interquartile range, 3.3–5.1; P < 0.001). By comparing the tumor and corresponding nontumor liver tissue obtained from the same patient, a higher GEP transcript level was observed in the tumor in the majority of HCC patients [49 (89.1%) of 55].

The fact that the expression level in HCCs was higher than that in the adjacent nontumor liver tissues could be interpreted as either GEP up-regulation in HCCs or GEP down-regulation in nontumor liver tissues. To distinguish the two situations, 22 normal liver tissues were examined. GEP transcript was expressed at a low level in normal livers (median, 4.0; interquartile range, 3.3–4.4), which was significantly lower than that in the HCCs (P < 0.001), but there was no significant difference in GEP expression between normal liver tissues and the adjacent nontumor liver tissues (median, 4.4; range, 3.3–5.1).

**Table 1** GEP protein expression in human liver samples

<table>
<thead>
<tr>
<th>GEP protein expression score</th>
<th>HCC (n = 110)</th>
<th>Nontumor (n = 110)</th>
<th>Normal (n = 22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (negative signal)</td>
<td>25 (22.7%)</td>
<td>72 (65.5%)</td>
<td>22 (100%)</td>
</tr>
<tr>
<td>1 (weak signal)</td>
<td>17 (15.5%)</td>
<td>37 (33.6%)</td>
<td>0</td>
</tr>
<tr>
<td>2 (intermediate signal)</td>
<td>22 (20.0%)</td>
<td>1 (0.9%)</td>
<td>0</td>
</tr>
<tr>
<td>3 (strong signal)</td>
<td>46 (41.8%)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table 2** Clinicopathological features of HCC in relation to GEP expression

<table>
<thead>
<tr>
<th>Clinicopathological variables</th>
<th>Weak (score 0–2)</th>
<th>Strong (score 3)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Venous infiltration</td>
<td>37</td>
<td>13</td>
<td>0.002*</td>
</tr>
<tr>
<td>Absence</td>
<td>27</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>Presence</td>
<td>30</td>
<td>13</td>
<td>0.048*</td>
</tr>
<tr>
<td>Tumor size</td>
<td>34</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>Small (≤5 cm)</td>
<td>14</td>
<td>18</td>
<td>0.049*</td>
</tr>
<tr>
<td>Large (&gt;5 cm)</td>
<td>50</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Intrahepatic recurrence in the first year</td>
<td>14</td>
<td>18</td>
<td>0.050</td>
</tr>
<tr>
<td>Yes</td>
<td>28</td>
<td>14</td>
<td>0.105</td>
</tr>
<tr>
<td>No</td>
<td>33</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>pTNM stage</td>
<td>55</td>
<td>34</td>
<td>0.113</td>
</tr>
<tr>
<td>Early stage (I–II)</td>
<td>9</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Late stage (III–IV)</td>
<td>34</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
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<tr>
<td>Male</td>
<td>26</td>
<td>12</td>
<td>0.300</td>
</tr>
<tr>
<td>Female</td>
<td>38</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>58</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>Young (≤ median, 52)</td>
<td>6</td>
<td>4</td>
<td>1.000</td>
</tr>
<tr>
<td>Elderly (&gt; median, 52)</td>
<td>34</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>Serum AFP level</td>
<td>10</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Low (≤20 ng/ml)</td>
<td>26</td>
<td>12</td>
<td>0.114</td>
</tr>
<tr>
<td>High (&gt;20 ng/ml)</td>
<td>38</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>HBV association</td>
<td>58</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>Positive for HBsAg</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative for HBsAg</td>
<td>4</td>
<td></td>
<td></td>
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</tbody>
</table>

Abbreviations: AFP, α-fetoprotein; HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen.
between the normal livers and the nontumor liver tissues ($P = 0.173$; Fig. 1B).

**Granulin–Epithelin Precursor Protein Expression.** To examine whether the GEP protein levels correlate with the change at transcript levels, immunohistochemical staining of GEP was performed on all of the 110 pairs of HCCs and their corresponding nontumor liver tissues, as well as the 22 normal livers. GEP protein staining was observed in tumor cytoplasm (Fig. 2). With the immunohistochemical method, the GEP protein level in the HCCs was found significantly higher than that of the nontumor liver tissues and normal livers (median scores of 2, 0, and 0, respectively; $P < 0.001$; Table 1). The majority of HCCs revealed the presence of GEP protein signals [75 (77.3%) of 110], which were predominantly intermediate to strong. The GEP protein was detectable only in a portion of nontumor liver tissues [38 (34.5%) of 110], the signals of which were mostly weak, and not detectable in the normal liver tissues [0 (0%) of 22]. The majority of HCCs demonstrated up-regulation of GEP protein compared with their corresponding nontumor liver tissues [79 (71.8%) of 110].

The GEP RNA levels (by quantitative measurement) and protein levels (by semiquantitative scoring) were compared. The HCCs demonstrated significant correlation of the GEP RNA level with the protein level ($r = 0.365, P = 0.006$). Notably, the RNA and protein levels in nontumor liver tissues and normal livers did not show significant correlation.

**Fig. 1** GEP transcript levels in human liver samples. A, correlation of microarray data and real-time quantitative RT-PCR data in HCC tissues. B, quantitative RT-PCR data presented in boxplot. A significantly higher GEP level in HCC compared with nontumor liver tissue and normal liver was observed. The top and bottom horizontal lines of the box, the 25th and 75th percentiles, respectively. The lines within the box, the median values. The top and bottom horizontal bars, data within 1.5 times the interquartile range.

**Fig. 2** Immunohistochemical staining of GEP protein in human liver samples. A, HCC with protein signal score 3. B, nontumor liver tissue with protein score 0. C, normal liver tissue with protein score 0.
Clinicopathological Features of Hepatocellular Carcinoma with Strong GEP Expression. The GEP expression levels in HCCs were further analyzed for clinicopathological significance. The HCCs were categorized into “weak” and “strong” expression groups according to the median score value. HCCs with scores ≤ median (scores 0–2) were categorized into the weak expression group, and HCCs with scores > median (score 3) were categorized into the strong expression group. Strong GEP protein expression was significantly associated with venous infiltration, large tumors (>5 cm), and intrahepatic recurrence in the first year (P < 0.05; Table 2). A strong GEP protein level was marginally associated with the absence of tumor capsule (P = 0.05). However, the GEP expression level was not significantly associated with the pTNM stages, gender, age, serum α-fetoprotein level, or hepatitis B virus association (assessed by serum hepatitis B surface antigen).

Granulin–Epithelin Precursor Function in Cell Proliferation. The Hep3B parental cells expressed a high level of endogenous GEP transcript by quantitative RT-PCR (7.2, compared with the median value of 6.1 in HCCs, 4.4 in nontumor liver tissues, and 4.0 in normal livers). The cells were transfected with different constructs and examined for the GEP protein level difference. To examine whether growth factors from serum would affect the transfectants differently, we compared the growth rate of transfected cell lines in serum-containing (10% serum) and serum-starved (0% serum) conditions. For Hep3B cells transfected with the antisense fragment, a reduced GEP protein level was observed in both serum-containing and serum-starved conditions (Fig. 3A and B; Table 3). For Hep3B full-length transfectants, a higher GEP protein level was observed in both serum-containing and serum-starved conditions. The sense control and empty vector control in Hep3B transfectants demonstrated a similar GEP level as their respective parental cells in both serum conditions.

The cell proliferation rate was also examined in serum-containing and serum-starved conditions (Fig. 3C; Table 3). Only the antisense transfectants resulted in a significant decrease in cell proliferation by 74 and 55% in serum-containing and serum-starved conditions, respectively (P < 0.01). The full-length, sense, and empty vector transfectants demonstrated a similar growth rate as the parental cell line. The cell activity was measured by the MTT assay via the mitochondrial activity (Fig. 3D). The antisense transfectants demonstrated marked reduction of cell activity in both serum-containing and serum-starved conditions (P < 0.05). The full-length, sense, and vector transfectants all demonstrated a similar cell activity with the parental cell line in both conditions.

Granulin–Epithelin Precursor Function in Cell Invasion. The cell invasion ability was investigated with the Matrigel cell invasion chamber (Fig. 4A). The antisense transfectants showed marked reduction in cell invasion ability, by 81%...
compared with the empty vector control (5.9 ± 0.5 and 30.7 ± 1.0 cells per field, respectively; P < 0.01).

Granulin–Epithelin Precursor Function in Tumorigenicity and Metastasis. The aggressiveness and metastasis potential of cancer cells in vitro was assessed by anchorage-independent growth in soft agar for their ability to form colonies (Fig. 4B). Hep3B antisense transfectants showed a 55% reduction in the anchorage-independent growth ability compared with the empty vector control (5.6 ± 0.4 and 12.4 ± 2.0 colonies per field, respectively; P < 0.05). The tumorigenic potential was assessed in vivo in the athymic nude mice (Fig. 4C). The Hep3B antisense transfectants developed small tumors only in 3 of 5 mice examined, whereas the empty vector transfectants developed large tumors in all of the 5 mice tested. The tumor weight of the antisense group was significantly reduced by 87% compared with the vector control group (0.3 ± 0.4 and 2.3 ± 0.7 g, respectively; P < 0.001).

DISCUSSION

The advance of the microarray technology has provided a new approach to evaluate a large number of genes efficiently. Genome-wide expression profiling provides a comprehensive picture to understand the complex biological behavior, development, and progression of cancer. We have demonstrated in the earlier microarray study that GEP was differentially expressed in HCCs compared with nontumor liver tissues (5). With the secretory protein nature, the genes with higher expression levels in tumors have the potential to serve as serum markers for disease diagnosis or prognosis. Six of these genes (GRN, PLTP, SEMG2, AFP, STC1, and TIMP1) were designated as secretory proteins. Importantly, AFP (α-fetoprotein), a widely used HCC serum marker today, is among these 6 secretory proteins. The feasibility of the current rationale for identification of tissue markers is thus confirmed. Chromosome gain at 1q, 8q, and 17q are frequently detected in HCC and these chromosome regions may contain important oncogenes/growth factors. GEP is the only one among the 6 genes that is located at chromosome 17q21.32, the frequently amplified regions, and is a novel growth factor. Therefore, GEP has drawn the focus of further investigation, although the average transcript level difference between the tumor and nontumor liver tissues by microarray was less than 2-fold. In this study, we validated that expression of GEP was unique in HCCs, whereas up-regulation of GEP was uncommon in nontumor liver tissues and in normal livers. The functional study also revealed the potential of GEP to serve as a therapeutic target for treatments such as specific gene silencing by using the antisense approach (20) or RNA interference (21). The GEP promoter will also be valuable for HCC suicide gene therapy (22).

This is the first report to reveal GEP overexpression in HCC. Both the RNA and protein levels in the HCCs were significantly higher than those in the nontumor liver tissues and normal livers. Significant correlation of RNA with protein levels was observed in HCCs but not in other tissues. The majority of nontumor liver tissues and normal liver tissues showed an undetectable protein level by the immunohistochemical method (median score of 0), explaining why the semiquantitative protein data failed to correlate with the quantitative RNA data in these liver tissues except HCCs. This study is also the first report on quantitative real-time RT-PCR assay of the GEP transcript level in human cancer, and reveals the positive correlation of GEP RNA with protein levels in HCCs.

Strong GEP expression was associated with aggressive HCC features including large tumor size, presence of venous infiltration, and early intrahepatic recurrence. Large tumor size is associated with a late stage of disease (23) and disease recurrence (24–26). Venous infiltration is a microscopic feature that demonstrates the presence of clusters of cancer cells in the vascular space lined by endothelial cells (26–27), and this microscopic metastasis is predictive of intrahepatic metastasis and poor prognosis (27–29). Therefore, the involvement of GEP in the aggressive HCCs suggests that GEP may play a pivotal role in HCC progression.

Transfection studies demonstrated that GEP positively regulated the cell proliferation rate, cell activity by MTT, cell invasion ability, colony-forming ability in anchorage-independent environment, and the tumorigenic potential. Notably, a decreased GEP level by antisense transfection decreased the proliferation rate, although an increased GEP level by full-length transfection could not further increase the growth rate. This may be because the endogenous GEP level in Hep3B is already at a high level (7.2, compared with the median value of 6.1 in HCCs, 4.4 in nontumor liver tissues, and 4.0 in normal livers. Significant correlation of RNA with protein levels in HCCs. Both the RNA and protein levels in the HCCs were significantly higher than those in the nontumor liver tissues and normal livers. Significant correlation of RNA with protein levels was observed in HCCs but not in other tissues. The majority of nontumor liver tissues and normal liver tissues showed an undetectable protein level by the immunohistochemical method (median score of 0), explaining why the semiquantitative protein data failed to correlate with the quantitative RNA data in these liver tissues except HCCs. This study is also the first report on quantitative real-time RT-PCR assay of the GEP transcript level in human cancer, and reveals the positive correlation of GEP RNA with protein levels in HCCs.

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Strong GEP expression was associated with aggressive HCC features including large tumor size, presence of venous infiltration, and early intrahepatic recurrence. Large tumor size is associated with a late stage of disease (23) and disease recurrence (24–26). Venous infiltration is a microscopic feature that demonstrates the presence of clusters of cancer cells in the vascular space lined by endothelial cells (26–27), and this microscopic metastasis is predictive of intrahepatic metastasis and poor prognosis (27–29). Therefore, the involvement of GEP in the aggressive HCCs suggests that GEP may play a pivotal role in HCC progression.
livers). Thus, a forced GEP level increased by GEP full-length transfection cannot promote the cell proliferation rate to a further extreme. Additional studies should involve hepatoma cell lines with intermediate to low levels of endogenous GEP. Alternatively, other factors may be necessary together with GEP to exert the role on cell proliferation and, therefore, increase of the GEP level alone cannot induce cell proliferation. Conversely, on serum deprivation, the ability of the increase of GEP level in the full-length transfectant was less apparent than in the serum-containing condition, suggesting that some other factors in the serum would be important for GEP protein up-regulation. Nonetheless, the functional data by antisense experiments further corroborated the clinical observations of strong GEP expression in large HCCs, in tumors with venous infiltration, and in association with early intrahepatic recurrence. Thus, GEP played a major role in hepatocarcinogenesis, contributing to the development of different tumor stages including tumor growth, invasion, and metastasis.

The exact mechanism for GEP up-regulation and how GEP executes the biological function is unknown, because the GEP-associated genes and/or pathways are not well characterized in HCC. Nonetheless, GEP expression has been reported in other aggressive cancers (30, 31) with a functional role in cell growth regulation (10, 32, 33), wound-healing process (34), and murine development (35). GEP has been reported to activate p44/42 mitogen-activated protein kinase in the extracellular regulated kinase pathway, phosphatidyl inositol-3-kinase/AKT-protein kinase B pathway, and focal adhesion kinase in the adhesion/motility pathway in different cell types (36–39). Activation of p44/42 mitogen-activated protein kinase/extracellular regulated kinase, phosphatidyl inositol-3-kinase, and focal adhesion kinase pathways has been reported to function in HCC progression (40–42). Therefore, control of HCC growth, invasion and metastasis by GEP could be mediated through these pathways. A decreased level of p44/42 mitogen-activated protein kinase phosphorylated form was observed in the GEP antisense transfectants.6 Our pilot study indicated that control of hepatoma growth would be mediated through the activation of p44/42 mitogen-activated protein kinase in the extracellular regulated kinase pathway. We have also commenced to examine GEP-regulated genes by the microarray approach to delineate the exact mechanism in hepatoma.

In summary, we demonstrated that GEP overexpression is specific to HCCs, with a minimal level of expression in the nontumor liver tissues and normal livers. The functional data agreed well with the clinical observation, in which strong GEP expression contributed to HCC growth, invasion, and metastasis. All of these findings suggest that GEP is an important factor in hepatocarcinogenesis, and that GEP has the potential to serve as a tumor marker and therapeutic target. Further investigation into the mechanism of GEP up-regulation and the GEP pathway is needed.

\[6\] S. Y. Wong and S. T. Cheung, unpublished data.

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