Human G-protein gamma 7 in extrahepatic cholangiocarcinoma and its clinicopathological significance

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BACKGROUND AND OBJECTIVES: Several studies have found a down-regulated G-γ 7 gene in gastrointestinal tract cancers. We evaluated the expression and clinicopathological significance of the human G protein γ 7 (G-γ 7) in human extra-hepatic cholangiocarcinoma (EHCC).

METHODS: The expression of G-γ 7 expression was studied in 21 patients with EHCC. G-γ 7 mRNA expression was tested by using RealTime reverse transcription polymerase chain reaction (RT-PCR). To visualize the localization of G-γ 7, an immunohistochemistry study was also performed. The G-γ 7 expression was compared among cancer tissues, peri-cancerous bile duct tissues and normal bile duct tissues. The clinicopathological significance of G-γ 7 expression was also studied.

RESULTS: Expression of G-γ 7 mRNA and protein were significantly lower in EHCC tissue than in peri-cancerous bile duct tissue and normal bile duct tissues. G-γ 7 mRNA and protein expression were significantly lower in poorly differentiated EHCC tissues than in moderate differentiated and well differentiated EHCC tissues (P<.01). There was no significant correlation between G-γ 7 expression and host factors such as age, gender, clinical staging or the status of preoperative hepatic function.

CONCLUSIONS: EHCC has a down-regulated expression of G-γ 7. Reduced expression of G-γ 7 is associated with the histological grade of EHCC and may prove to be a useful marker for predicting the prognosis of human EHCC.

Cholangiocarcinoma is rising in clinical importance because of its increasing incidence, sub-optimal response to therapy, and poor prognosis. Recent investigations into the underlying molecular mechanisms involved in cholangiocarcinogenesis and tumor growth have contributed greatly to our understanding of this disease. Conversion from normal to malignant bile epithelium probably requires a stepwise accumulation of successive genetic abnormalities involving a variety of molecular defects of both oncogenes and tumor suppressor genes. However, the molecular pathogenesis of cholangiocarcinoma is still largely unknown.

The guanine nucleotide-binding proteins (G proteins) play a key role in cell signaling. Its β and γ subunit control the signals involved in cell growth. The G-γ 7 gene is widely distributed in the signal transduction pathways, and G-γ 7-coupled G proteins may contribute to carcinogenesis in many cancers. Several studies have found a down-regulated G-γ 7 gene in gastrointestinal tract cancers, including esophageal, gastric, pancreatic, and colorectal cancers. To our knowledge, the expression of G-γ 7 hasn’t been confirmed in extra-hepatic cholangiocarcinoma. In this study, we evaluated the expression of G-γ 7 in 21 extra-hepatic cholangiocarcinoma (EHCC) patients. The expression of G-γ 7 was also compared among cancer tissues, peri-cancerous normal bile duct tissue, and normal bile duct tissues. The association of G-γ 7 and the histological grade of EHCC were also investigated.

METHODS
Twenty-one patients (14 male, 7 female) with distal EHCC who underwent surgery at the Changhai Hospital, Shanghai Second Military Medical University, were studied. Mean age was 58.8 years. All patients un-
derwent pancreaticoduodenectomy (Whipple procedure) for primary tumors not associated with primary sclerosing cholangitis. After the surgery, the tumor and corresponding non-tumor tissues were immediately frozen in liquid nitrogen and stored at –80°C until use. Percanerous normal bile duct tissue was taken from the area within 2 cm from the edge of cancer. Normal bile duct tissue was taken from the area at least 5 cm away from the edge of cancer. Clinical staging: 10 patients were stage I-II, 11 patients were stage I-II. The study has approved by the Specialty Committee on Ethics of Biomedicine, the Second Military Medical University.

**RNA extraction and real time reverse-transcription (RT)-PCR analysis**

Tissue samples were taken immediately after surgery and stored at –80°C. RNA was extracted using the TRIzol reagent (TaKaRa Bio Inc, Japan) according to the manufacturer’s recommendations. The RNA was dissolved to 1(µL) using diethylpyrocarbonate-treated water and stored at –80°C until use.

Primer sequence was designed according to published mRNA sequences of G-γ7 gene (GenBank ID: NM-016373: 128bp 5’-TGTCAGCCACTAACATAGCAGGCAGGGCCGGGAAGCTGTGGAACAGCTACGCATAGAAGCAGGATGACGCCGCACTACGGTCTCAAAGCGCGCTCTGACCTCATGAGCTACTGTGAGCAACATGCC 3’). The oligonucleotide primer pairs for G-γ7 were synthesized (ShengGong Inc, China) (Sense primer: 5’-TGTCAAGGCACTAACATAGCAGGCAGGGCCGGGAAGCTGTGGAACAGCTACGCATAGAAGCAGGATGACGCCGCACTACGGTCTCAAAGCGCGCTCTGACCTCATGAGCTACTGTGAGCAACATGCC 3’; antisense primer: 5’-GGCATGTGGTCTCAAGTATAGC-G3’).

G-γ7 mRNA extracted from 100mg tissues was prepared using the RNeasy Kit (Huashun, China), followed by reverse transcription into cDNA with oligo-dT primers (Takara Bio Inc, Japan). PCR was conducted in GeneAmp PCR system 2400 (GeneAmp, USA). A reaction mix of 40 µL was prepared, incubated at 42°C for 60min, reacted at 70°C for 6min and then denaturated of reverse transcriptase. PCR was performed, in a total mix of 1 µL cDNA, dNTP, forward and reverse primers, and Taq polymerase (TakaRa Bio Inc, Japan). Positive and negative controls were included in all runs. The PCR was performed using a method described previously.

Standard recombinant plasmid preparation: PCR product was cloned into the pMD18-T vector (Takara Bio Inc, Japan). The clones were inoculated into 100 µL Escherichia coli DH5α competent cells. White colonies were selected and screened by PCR. Briefly, white colonies were picked, incubated in Amp+ LB broth overnight at 37°C, 200 rpm/min. Plasmid DNA were extracted and purified according to recommendation of manufacturer (miniBest plasmid purification kit Ver 2.0, Takara Bio Inc, Japan). Followed by PCR amplification and agarose gel visualizing, the concentration of plasmid DNA was calculated as 251.4 ng/µL. The plasmid DNA was diluted as 10-2, 10-3, 10-4, 10-5, 10-6 ng/µL to be used as standard benchmark.

Real time RT-PCR: PCR reactions containing SYBR-green were amplified on a Corbett Real Time PCR machine (Roche Diagonostics, USA). The 20 µL Tag reaction mix included G-γ7 forward and reverse primers and 1 µL standard plasmid or tissue. Real-time quantification of cDNA was performed. Lngtclycer Version 5.32 was run to analyze the parameters to calculate the original concentration of the template.

**Table 1. The expression of G-γ7 mRNA in EHCC.**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sex</th>
<th>Age</th>
<th>Histological grade</th>
<th>G-γ7 mRNA (x10^6 ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>70</td>
<td>Poorly differentiated</td>
<td>0.7646</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>74</td>
<td>Moderately differentiated</td>
<td>1.8030</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>73</td>
<td>Poorly differentiated</td>
<td>1.3230</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>50</td>
<td>Poorly differentiated</td>
<td>0.9248</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>59</td>
<td>Well differentiated</td>
<td>3.7920</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>50</td>
<td>Poorly differentiated</td>
<td>1.4950</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>38</td>
<td>Moderately differentiated</td>
<td>2.6140</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>69</td>
<td>Moderately differentiated</td>
<td>2.0312</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>65</td>
<td>Well differentiated</td>
<td>2.1215</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>70</td>
<td>Poorly differentiated</td>
<td>1.2112</td>
</tr>
<tr>
<td>11</td>
<td>M</td>
<td>47</td>
<td>Moderately differentiated</td>
<td>1.9586</td>
</tr>
<tr>
<td>12</td>
<td>F</td>
<td>43</td>
<td>Moderately differentiated</td>
<td>1.8959</td>
</tr>
<tr>
<td>13</td>
<td>M</td>
<td>46</td>
<td>Poorly differentiated</td>
<td>1.5642</td>
</tr>
<tr>
<td>14</td>
<td>F</td>
<td>62</td>
<td>Poorly differentiated</td>
<td>1.2456</td>
</tr>
<tr>
<td>15</td>
<td>M</td>
<td>56</td>
<td>Well differentiated</td>
<td>2.0012</td>
</tr>
<tr>
<td>16</td>
<td>M</td>
<td>70</td>
<td>Poorly differentiated</td>
<td>0.9910</td>
</tr>
<tr>
<td>17</td>
<td>M</td>
<td>64</td>
<td>Moderately differentiated</td>
<td>1.6258</td>
</tr>
<tr>
<td>18</td>
<td>M</td>
<td>72</td>
<td>Well differentiated</td>
<td>2.8952</td>
</tr>
<tr>
<td>19</td>
<td>M</td>
<td>47</td>
<td>Poorly differentiated</td>
<td>1.1469</td>
</tr>
<tr>
<td>20</td>
<td>M</td>
<td>58</td>
<td>Well differentiated</td>
<td>3.4216</td>
</tr>
<tr>
<td>21</td>
<td>M</td>
<td>52</td>
<td>Well differentiated</td>
<td>2.9626</td>
</tr>
</tbody>
</table>
immunohistochemical staining was performed with 1.0 mM EDTA buffer pH 8.0 for 10 min in a microwave oven followed by a 20-min cooling down. In order to block nonspecific antibody binding, tissue samples were incubated with 10% goat serum in PBS for 30 min. Primary polyclonal rabbit G-γ7 antibody was used at a 1:50 dilution overnight at 4°C. Rabbit serum used as an isotype control at similar concentrations. This was followed by incubation with Envision HRP labeled polymer conjugated to goat-anti-rabbit immunoglobulins (Dako) for 30 min at room temperature. Staining development was performed with DAB with timed monitoring using a positive control sample. The slides were then counterstained with hematoxylin, dehydrated, cleared, and mounted. These sections were read by a pathologist.

The clinical pathological data variables were available for evaluation. The data were compared between G-γ7-positive and G-γ7-negative patients. Spearman (nonparametric) correlation was used to correlate G-γ7 expression with age, gender, tumor histological grade, tumor stage and the status of preoperative hepatic function. Fisher’s exact test was used in data enumeration. The F-test was used in data measurement. The basic significance level was at P<.05 and all data was analyzed using SPSS statistical software (Version 11.0; SPSS Inc., Chicago, IL).

RESULTS
The histological grading of extra-hepatic cholangiocarcinoma was as follows: 6 patients were Grade I (well differentiated); 6 were Grade II (moderately differentiated), and 9 were Grade III (poorly differentiated).

Gγ7 mRNA expression
EHCC samples expressed G-γ7 mRNA at significantly lower levels than peri-cancerous bile duct tissues and normal bile duct tissues. The average level of G-γ7 mRNA was 1.9000±0.8317×10⁶ ng/µL in EHCC tissues, 3.5920±0.8125×10⁶ ng/µL in peri-cancerous bile duct tissues, and 3.4799±0.4463 ng/µL in normal bile duct tissue samples (Tables 1, 2). Patient demographic information and histological grading are presented in Table 1.

Immunohistochemical staining
As shown in Figure 1, cells from five normal bile duct tissues have strong expression of cytoplasmic protein. In 21 primary EHCC tissues analyzed by immunohistochemistry staining, G-γ7 was weakly or moderately expressed in 10 of the EHCC tissue samples. The positivity was linked to the tumor cells. Eleven EHCC samples had a negative staining of cytoplasmic protein (Figure 2). The negativity expression rate was

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**Table 2. Quantitative comparison of G-γ mRNA expression.**

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>mRNA (10⁶ ng/µL)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>EHCC</td>
<td>1.9000±0.8317</td>
<td>.001*</td>
</tr>
<tr>
<td>Peri-cancerous bile duct</td>
<td>3.5920±0.8125</td>
<td>.002*</td>
</tr>
<tr>
<td>Normal bile duct tissues</td>
<td>3.4799±0.4463</td>
<td>.0069*</td>
</tr>
</tbody>
</table>

*EHCC compared with peri-cancerous bile duct tissue *EHCC compared with normal bile duct tissue *Peri-cancerous bile duct tissues compared with normal bile duct tissues.
G-protein gamma 7

52.38% among EHCC tissue samples. Moreover, for these 11 negative samples, 1 was from a well-differentiated EHCC (1/6, 16.67%), 2 were from moderately differentiated EHCC (2/6, 33.33%), and 8 were from poorly differentiated EHCC (8/9, 88.89%).

G-γ7 mRNA expression and clinicopathological data

The clinicopathological features analyzed in relation to the G-γ7 expression status are given in Table 3. Spearman analysis found that expression of G-γ7 mRNA had a significant correlation with histological grading. The correlation coefficients were –0.911 and –0.506, respectively (P<.0001 and P=.008, respectively). There was no significant correlation between G-γ7 expression and host factors such as age, gender, clinical staging or the status of preoperative hepatic function (P>.05). G-γ7 expression was significantly lower in poorly differentiated EHCC than in moderately and well-differentiated EHCC tissues (P<.05).

DISCUSSION

The identification of the change of gene expression in tumor tissues and non-tumor tissues is fundamental for understanding its biological mechanisms. G proteins are essential molecules in signal transduction pathways. Studies showed that G-γ7 has specific regulatory effects on adenylate cyclase signal transduction, may stimulate receptor nucleotide exchange, and plays an important role in intracellular signal transduction and controls growth signals in cells. Down-regulation of G-γ7 has been proved in several digestive organ cancers. In the present study, we studied the expression of G-γ7 and its clinical significance in EHCC.

Intrahepatic cholangiocarcinoma (IHCC) and extrahepatic cholangiocarcinoma are two closely related, but biologically unique neoplastic processes. This was supported by a prior study of methylation profiles of tumor suppressor genes in IHCC and EHCC. Study also revealed that p27 expression decreased progressively from proximal to distal in the biliary tree and correlated with location-related differences in outcome. Cyclin D1 and Bcl2 overexpression also varied in the bile duct tissue according to anatomic site. In the present study, we demonstrated that the expression of G-γ7 mRNA and its protein was strongly suppressed in EHCC tissues compared with normal bile duct tissues and peri-cancerous normal bile duct tissues. More than 50 per cent of EHCC tissues had a negative expression of G-γ7 by immunohistochemical study. The reduced expression of G-γ7 was also confirmed by prior study in IHCC, which indicate that G-γ7 may be the key point in the pathogenesis of IHCC and EHCC, and IHCC and EHCC may partially share a common molecular pathogenesis. The decreased expression of G-γ7 has also been confirmed in pancreatic, esophageal and colon cancer tissues, further support that this is a common phenomenon in the process of tumorgenesis. Reduced expression of G-γ7 is also correlated with loss of heterozygosity (LOH) and reduced transduction of growth inhibition signal with cell contact. As a result, G-γ7 has the potential to become the common target of genetic therapy to digestive tract cancers.

In this study, we found that the expression of G-γ7 mRNA was not associated with patient age, gender, and preoperative liver function, but was associated with histological staging of the cancer. The expression of G-γ7 mRNA was significantly lower in poorly differentiated EHCC than in moderately differentiated EHCC (P=.001). There was also statistically significant difference of G-γ7 expression in moderately differentiated and well differentiated cholangiocarcinoma (P=.020). Decreased expression of G-γ7 protein has also been found in poorly differentiated IHCC than in moderately differentiated IHCC.

Table 3. Correlation of G-γ7 expression with clinic-pathological characteristics.

<table>
<thead>
<tr>
<th>Clinicopathological characteristics</th>
<th>Case (No.)</th>
<th>G-γ7 (Ct value) (x10^-6)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;50y</td>
<td>14</td>
<td>2.0000±0.9342</td>
<td>.362</td>
</tr>
<tr>
<td>&lt;50y</td>
<td>7</td>
<td>1.7000±0.5600</td>
<td>.393</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>14</td>
<td>1.7000±0.5981</td>
<td>.950</td>
</tr>
<tr>
<td>Female</td>
<td>7</td>
<td>2.0000±0.9263</td>
<td>.850</td>
</tr>
<tr>
<td>Histological grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>6</td>
<td>1.2000±0.2614</td>
<td>.850</td>
</tr>
<tr>
<td>Moderately differentiated</td>
<td>6</td>
<td>1.9000±0.9712</td>
<td>.547</td>
</tr>
<tr>
<td>Well differentiated</td>
<td>9</td>
<td>2.9000±0.7041</td>
<td>.455</td>
</tr>
<tr>
<td>Clinical Stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I + II</td>
<td>10</td>
<td>1.8000±0.7913</td>
<td>.547</td>
</tr>
<tr>
<td>III + IV</td>
<td>11</td>
<td>1.9000±0.6551</td>
<td>.850</td>
</tr>
</tbody>
</table>

*Poorly-differentiated EHCC compared with moderately-differentiated EHCC. **Moderately-differentiated EHCC compared with well-differentiated EHCC.
original research report

also revealed that the expression of G-γ7 can induce
the expression of p27kip1, the expression of p27kip1 is
known to be related to survival in patients with various
cancers, and the absent p27kip1 expression independ-
ently predicted poor outcome. As a result, reduced
G-γ7 expression may through the reduced expression
of p27 to affect the outcome of EHCC and IHCC.
Since low expression of G-γ7 is associated with poor
histological grading, G-γ7 might be also a predictive
factor for disease progression and prognosis.

In conclusion, expression of G-γ7 mRNA was
down-regulated in EHCC tissues and was clinically
associated with its histological grade. The G-γ7 ex-
pression status may be used to predict the prognosis
of human EHCC. Although the precise function of G-
γ7 in the pathogenesis of EHCC remains unclear, our
discovery of aberrant expression of G-γ7 in EHCC
and its association with histological grade of EHCC
make it an interesting gene that warrants further in-
vestigation.

Author contribution:
Mei Wang and Biao Gong contributed equally. Dr. Wang
designed and conducted the study, while Dr. Gong selected
the cases and collected the samples. Li Yongmei did the RT-
PCR process and Yajie Wang monitored all the courses.

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