Epidermal Growth Factor Gene Functional Polymorphism and the Risk of Hepatocellular Carcinoma in Patients With Cirrhosis

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Context Overexpression of epidermal growth factor (EGF) in the liver induces transformation to hepatocellular carcinoma in animal models. Polymorphisms in the EGF gene modulate EGF levels.

Objective To assess the relationship among human EGF gene single-nucleotide polymorphism, EGF expression, and risk of hepatocellular carcinoma.

Design, Setting, and Participants Molecular mechanisms linking the 61*G allele polymorphism to EGF expression were examined in human hepatocellular carcinoma cell lines and human liver tissue. A case-control study involving 207 patients with cirrhosis was conducted at the Massachusetts General Hospital (1999-2006) and a validation case-control study involving 121 patients with cirrhosis was conducted at Hôpital Paul Brousse (1993-2006). Restriction fragment-length polymorphism was used to determine the EGF gene polymorphism genotype. Logistic regression analysis was used to assess the association between the EGF polymorphism and hepatocellular carcinoma risk.

Main Outcome Measures Mechanisms by which the EGF gene polymorphism modulates EGF levels and associations among EGF gene polymorphism, EGF levels, and hepatocellular carcinoma.

Results Transcripts from the EGF 61*G allele exhibited more than a 2-fold longer half-life than those from the 61*A allele, and EGF secretion was 2.3-fold higher in G/G hepatocellular carcinoma cell lines than A/A cell lines. Serum EGF levels were 1.8-fold higher in G/G patients than A/A patients, and liver EGF levels were 2.4-fold higher in G/G patients than A/A patients. Among the 207 patients with cirrhosis in the Massachusetts study population, 59 also had hepatocellular carcinoma. Analysis of the distribution of allelic frequencies revealed that there was a 4-fold odds of hepatocellular carcinoma in G/G patients compared with A/A patients in the Massachusetts study population (odds ratio, 4.0; 95% confidence interval [CI], 1.6-9.6; P = .002). Logistic regression analysis demonstrated that the number of copies of G was significantly associated with hepatocellular carcinoma after adjusting for age, sex, race, etiology, and severity of cirrhosis (G/G or A/G vs A/A; hazard ratio, 3.49; 95% CI, 1.29-9.44; P = .01). The significant association was validated in the French patients with alcoholic cirrhosis and hepatocellular carcinoma.

Conclusion The EGF gene polymorphism genotype is associated with risk for development of hepatocellular carcinoma in liver cirrhosis through modulation of EGF levels.

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EGF GENE POLYMORPHISM AND RISK OF HEPATOCELLULAR CARCINOMA

ABLE FOR SCREENING AND CHEMOPREVENTION HAVE BEEN PROPOSED AS ALTERNATIVE STRATEGIES.

SCREENING STRATEGIES FOR HIGH-RISK POPULATIONS INCLUDE ALPHA FETOPROTEIN MEASUREMENTS AND LIVER IMAGING. THESE TECHNIQUES ARE COSTLY AND ARE HINDERED BY SUBOPTIMAL SENSITIVITY AND SPECIFICITY. TO THIS END, IDENTIFICATION OF MOLECULAR MARKERS ASSOCIATED WITH AN INCREASED RISK OF HEPATOCELLULAR CARCINOMA WOULD BETTER DEFINE POPULATIONS AT HIGHEST RISK FOR HEPATOCELLULAR CARCINOMA AND MAY ADDITIONALLY DEFINE IMPORTANT THERAPEUTIC TARGETS FOR PREVENTION AND TREATMENT.

EPIDERMAL GROWTH FACTOR, FIRST ISOLATED IN 1962,4 HAS MANY BIOLOGICAL FUNCTIONS. IT STIMULATES PROLIFERATION AND DIFFERENTIATION OF EPIDERMAL AND EPITHELIAL TISSUES.6,7 EPIDERMAL GROWTH FACTOR IS A MITOGEN FOR ADULT AND FETAL HEPATOCYTES GROWN IN CULTURE,9,10 AND ITS EXPRESSION IS UP-REGULATED DURING LIVER REGENERATION.11 MOUNTING EVIDENCE SUPPORTS A ROLE FOR EGF IN MALIGNANT TRANSFORMATION AND TUMOR PROGRESSION.12 EPIDERMAL GROWTH FACTOR INDUCES TRANSFORMATION TO ANCHORAGE-INDEPENDENT GROWTH13 AND ENHANCES IN VITRO GROWTH OF HUMAN EPITHELIAL- AND MESENCHYMAL-DERIVED TUMORS.14 OVEREXPRESSION OF A SECRETED HUMAN EGF FUSION PROTEIN IN FIBROBLASTS ENHANCES THEIR TRANSFORMATION TO FIBROSARCOMAS.15 TRANSGENIC MICE WITH LIVER-TARGETED OVEREXPRESSION OF THE SECRETED EGF FUSION PROTEIN DEVELOP HEPATOCELLULAR CARCINOMA.16 GENE EXPRESSION PROFILES COMPARING NORMAL LIVER TISSUE WITH LIVER TUMORS IN THESE MICE SUGGEST A ROLE FOR AN AUTOCRINE MECHANISM DURING EGF-INDUCED HEPATOCARCINOGENESIS.17

SHAHBAZI ET AL18 IDENTIFIED A SINGLE-NUCLEOTIDE POLYMORPHISM INVOLVING A TO G TRANSITION AT POSITION 61 IN THE 5‘ UNTRANSLATED REGION OF THE EGF GENE (SNP rs4444903). THESE INVESTIGATORS DEMONSTRATED THAT IN VITRO CULTURES OF PERIPHERAL BLOOD MONONUCLEAR CELLS FROM INDIVIDUALS WITH THE G/G GENE TYPE SECRETE MORE EGF THAN PERIPHERAL BLOOD MONONUCLEAR CELLS FROM INDIVIDUALS WITH THE A/A GENE TYPE AND THAT THE G/G GENE TYPE WAS ASSOCIATED WITH AN INCREASED RISK OF DEVELOPING MALIGNANT MELANOMA COMPARED WITH THE A/A GENE TYPE. IN THIS STUDY, WE SOUGHT TO PERFORM A FUNCTIONAL ANALYSIS OF THIS EGF GENE SINGLE-NUCLEOTIDE POLYMORPHISM, DETERMINE ITS INFLUENCE ON SERUM AND LIVER EGF LEVELS, AND ASSESS ITS CORRELATION WITH RISK OF HEPATOCELLULAR CARCINOMA IN PATIENTS WITH CIRRHOSIS.

METHODS

CELL CULTURE

HEPATOCELLULAR CARCINOMA CELL LINES SNU-182, SNU-387, SNU-398, SNU-423, SNU-449, AND SNU-47519 WERE OBTAINED FROM AMERICAN TYPE CULTURE COLLECTION (ATCC, ROCKVILLE, MARYLAND). THE SK-HEP, PLC/PRF/5, HEPG2, AND HEP3B CELL LINES WERE PROVIDED BY BARRIE BODE, PhD (SAINT LOUIS UNIVERSITY, SAINT LOUIS, MISSOURI). THESE 10 CELL LINES REPRESENT ALL HEPATOCELLULAR CARCINOMA CELL LINES THAT ARE CURRENTLY COMMERCIAL AVAILABLE IN THE UNITED STATES. ADDITIONALLY, WE OBTAINED HUH-7 CELLS FROM JAKE LIANG, MD (NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES, BETHESDA, MARYLAND), AND FOCUS CELLS FROM JACK WANDS, MD (BROWN UNIVERSITY, PROVIDENCE, RHODE ISLAND). ALL THE CELL LINES WERE PROPAGATED IDENTICALLY IN DULBECCO MODIFIED EAGLE MEDIUM (4.5 mg/mL GLUCOSE, 2 mM L-GLUTAMINE) WITH 10% FETAL BOVINE SERUM (BOTH FROM MEDIA TECH CELLGRO, HERNDON, VIRGINIA), SUPPLEMENTED WITH 100 U/mL PENICILLIN AND 100 mg/mL STREPTOMYCIN (INVITROGEN, CARLSBAD, CALIFORNIA). CELLS WERE MAINTAINED AT 37°C IN A HUMIDIFIED INCUBATOR WITH 5% CO2 IN AIR. PRIMARY CULTURES OF HUMAN HEPATOCYTES WERE PREPARED AS PREVIOUSLY DESCRIBED.20

TISSUE AND CLINICAL INFORMATION


FOR VALIDATION OF EGF GENE SINGLE-NUCLEOTIDE POLYMORPHISM GENOTYPE RESULTS OBSERVED IN THE MASSACHUSETTS POPULATION, AN INDEPENDENT GROUP OF 121 FRENCH PATIENTS WITH ALCOHOLIC CIRRHOSIS SEEN AT HÔPITAL PAUL BROUSSE BETWEEN THE YEARS 1993 AND 2006 WAS GENOTyped USING BLOOD OR LIVER TISSUE AS APPROVED BY HÔPITAL PAUL BROUSSE CENTRE DE RESSOURCES BIOLGIQUES. FORTY-FOUR OF THESE PATIENTS HAD HEPATOCELLULAR CARCINOMA (CASES), AND THE REMAINING 77 PATIENTS SERVED AS CONTROLS. NEITHER SERUM NOR TISSUE WAS AVAILABLE FOR ANALYSIS FROM THIS GROUP. ETHNICITY WAS STUDIED BECAUSE SINGLE-NUCLEOTIDE POLYMORPHISM FREQUENCIES ARE KNOWN TO DIFFER BETWEEN ETHNIC GROUPS. ETHNICITY WAS SELF-CLASSIFIED BY EACH SUBJECT.

DNA EXTRACTION AND GENOTYPING OF EGF GENE

DNA WAS EXTRACTED FROM HEPATOCELLULAR CARCINOMA CELL LINES (1 X 106 CELLS) AND FORMALIN FIXED PARAFFIN-EMBEDDED TISSUE (THREE 10-µM SECTIONS PER EACH PATIENT CASE) USING THE MASTERPURE PuriFICATION (EPICENTER, MADISON, WISCONSIN). LYMPHOCYTES WERE ISOLATED FROM WHOLE BLOOD USING Histopaque1077 (SIGMA, ST LOUIS), FOLLOWED BY DNA ISOLATION AS DESCRIBED ABOVE. THE EGF POLYMORPHISM WAS ANALYZED IN DUPLICATE, INDEPENDENTLY BY 2 DIFFERENT INVESTIGATORS USING RESTRICTION FRAGMENT-LENGTH POLYMORPHISM AS DESCRIBED PREVIOUSLY.18 BRIEFLY, GENOMIC DNA WAS SUBJECTED TO POLYMERASE CHAIN REACTION (PCR) AMPLIFICATION (INITIAL DENATURATION AT 95°C FOR 5 MINUTES, FOLLOWED BY 35 CYCLES AT 95°C FOR 30 SECONDS, 51°C FOR 30 SECONDS, AND 72°C FOR 1 MINUTE WITH A FINAL EXTENSION STEP OF 7 MINUTES AT 72°C) TO AMPLIFY NUCLEOTIDE POSITIONS 78 TO +164 OF THE EGF GENE. THE FOLLOWING PRIMERS WERE USED: FORWARD-TGTCAC-TAAAGAAGGAGGT AND REVERSE-CTTACAGATTATTACAGCC. THE
25 µL PCR product was digested overnight with 5 units of AluI at 37°C, separated by electrophoresis in a 3% agarose gel and visualized by staining with ethidium bromide. AluI digestion of the 242 base pair (bp) PCR product containing the 61*A allele produced 15, 34, and 193 bp fragments, while digestion of the 61*A allele produced 15, 34, 91, and 102 bp fragments.

Real-Time PCR
Epidermal growth factor messenger RNA (mRNA) in hepatocellular carcinoma cell lines was measured by quantitative reverse transcription-PCR (LightCycler; Roche Diagnostics Corp, Indianapolis, Indiana). Cells were plated at 1 x 10^5 cells/mL in 10 mL of media in 10-cm plates and allowed to grow for 48 hours to reach log phase growth. Total RNA was extracted from each cell line using TRIzol (Invitrogen) and subsequently treated with DNase I (Promega, Madison). Two hundred fifty nanograms of total RNA from each sample was used to synthesize complementary DNA (cDNA) by single-strand reverse transcription (SuperScript III First-Strand Synthesis SuperMix for qRT-PCR; Invitrogen). All of the sample cDNAs were pooled together to create a quantitative standard control. The level of EGF mRNA present is expressed as the ratio of EGF mRNA present at each time point divided by its putative mRNA present at the time point at which mRNA levels had declined to 50% of their original levels. All of the reactions were performed in duplicate and the experiment was repeated to ensure accuracy. The following allele-specific primers were used: EGF 61*A forward: GCCCCAATCTCAAGGGTTGTA; EGF 61*G forward: GCCCCAATCCAGGGTTGTG; and reverse primer for both alleles: GCCAAAGGAAGCCACAGGAAAG. Similar studies were performed on primary cultures of human hepatocytes established from resected liver specimens from EGF 61A/G (heterozygous) patients. Hepatocyte cultures were established as previously described. 21

EGF and Phospho-EGF Receptor Enzyme-Linked Immunosorbent Assay
The phosphorylated EGF receptor was quantified using an enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, Minnesota). Epidermal growth factor protein was quantified using an enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, Minnesota). Conditioned media was collected for analysis and the supernatant was removed and stored at −80°C prior to use.

Statistical Methods
Comparisons of groups with regard to odds of hepatocellular carcinoma were made using a logistic regression model; age, sex, ethnicity, etiology of cirrhosis, and severity of cirrhosis were included as covariates in addition to genotype. Comparisons of EGF expression in cell lines and tissue/serum by number of copies of G were made using a
RESULTS

EGF Expression in Cell Lines

To analyze potential mechanisms by which the EGF gene polymorphism modulates EGF expression, a restriction fragment-length polymorphism strategy was used to genotype 12 human hepatoma cell lines for the presence of the A to G single-nucleotide polymorphism at position 61 of the EGF gene (Figure 1). Three of the cell lines proved to be the A/A genotype, 7 were G/G, and 2 were A/G. Because allelic variation in gene expression can result from differences in mRNA stability, real-time PCR with allele-specific primers was used to determine the stability of the different alleles after treatment with 5 µg/mL of actinomycin D. The PLC/PRF/5 and HepG2 cell lines were used for this experiment because they are heterozygous at the EGF gene polymorphism, which allows for stability of both types of EGF mRNA transcripts to be assessed in an otherwise identical environment.

The mRNA half-life for transcripts from the G allele was significantly longer than the half-life of A allele transcripts in both cell lines (P<.01; Figure 2). Greater stability of G allele compared with A allele transcripts was also observed in primary cultures of hepatocytes from patients heterozygous at the EGF gene polymorphism (P<.01; Figure 2). As summarized in Table 1, in accord with the greater stability of G allele mRNA, there was a nonsignificant increase in EGF mRNA with the number of copies of G alleles in the 12 human hepatocellular carcinoma cell lines (P=.06). More importantly, levels of intracellular EGF protein were significantly greater in cell lines with more copies of G in the genotype (P=.005), and cell lines secreted sig-
significantly more EGF into media with the more copies of $G$ in the genotype ($P = .002$).

**EGF Polymorphisms in Cirrhotic Patients**

Because EGF overexpression in the liver leads to development of hepatocellular carcinoma in mouse models, we examined whether the EGF gene polymorphism genotype correlated with risk for hepatocellular carcinoma in patients with cirrhosis by examining the EGF gene single-nucleotide polymorphism allelic distribution of all patients identified as having cirrhosis in the Massachusetts General Hospital Cancer Center Tumor bank.

Of these 207 patients, 59 also had hepatocellular carcinoma (Table 2). Clinically relevant factors of age, severity of cirrhosis, etiology of cirrhosis, sex, and ethnicity were evaluated. (Duration of cirrhosis cannot be determined with accuracy.) Sex and ethnic distribution were similar in A/A, A/G, and G/G patients, with the exception of Asians having more G copies than whites. The median age and etiology of cirrhosis were also similar among the 3 groups. The severity of cirrhosis as assessed by laboratory values used in the Child classification—total bilirubin, albumin, prothrombin time—were similar among the groups with the exception of slightly lower albumin levels in G/G patients relative to A/A patients.

Patients with an A/G genotype had a 2.4-fold and patients with a G/G genotype had 4.0-fold odds of developing hepatocellular carcinoma compared with patients with the A/A genotype (Table 3). The number of copies of $G$ was significantly associated with hepatocellular carcinoma ($P = .001$), further confirming this result. Logistic regression analysis demonstrates that number of copies of $G$ was significantly associated with hepatocellular carcinoma, after adjusting for age, sex, race, etiology, and severity of cirrhosis ($G/G$ plus A/G patients vs A/A patients hazard ratio, 3.49; 95% confidence interval [CI], 1.29-9.44; $P = .01$).

Epidermal growth factor and phosphorylated EGF receptor levels were measured in liver tissue specimens from 12 randomly selected patients of each genotype with cirrhosis (36 patients

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**Table 1. Epidermal Growth Factor (EGF) Expression Levels From Hepatocellular Carcinoma Cell Lines**

<table>
<thead>
<tr>
<th></th>
<th>A/A (n = 60)</th>
<th>A/G (n = 92)</th>
<th>G/G (n = 55)</th>
<th>P Valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/G vs A/A</td>
<td>.14</td>
<td>.02</td>
<td>.06</td>
<td></td>
</tr>
<tr>
<td>G/G vs A/A</td>
<td>.99</td>
<td>.03</td>
<td>.005</td>
<td></td>
</tr>
</tbody>
</table>

aWilcoxon-Mann-Whitney test.

**Table 2. General Characteristics of Massachusetts Study Group**

<table>
<thead>
<tr>
<th></th>
<th>A/A (n = 60)</th>
<th>A/G (n = 92)</th>
<th>G/G (n = 55)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean, y</td>
<td>56</td>
<td>54</td>
<td>55</td>
<td>.38</td>
</tr>
<tr>
<td>Men, No. (%)</td>
<td>44 (73)</td>
<td>67 (73)</td>
<td>43 (78)</td>
<td>.72</td>
</tr>
<tr>
<td>Race or ethnicity, No. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>56 (93)</td>
<td>78 (85)</td>
<td>42 (76)</td>
<td></td>
</tr>
<tr>
<td>Black</td>
<td>2 (3)</td>
<td>5 (6)</td>
<td>4 (7)</td>
<td>.62</td>
</tr>
<tr>
<td>Hispanic</td>
<td>2 (3)</td>
<td>5 (5)</td>
<td>3 (5)</td>
<td>.84</td>
</tr>
<tr>
<td>Asian</td>
<td>0 (0)</td>
<td>2 (2)</td>
<td>5 (9)</td>
<td>&lt;.01 a</td>
</tr>
<tr>
<td>Other</td>
<td>0 (0)</td>
<td>2 (2)</td>
<td>1 (2)</td>
<td>.62</td>
</tr>
</tbody>
</table>

**Table 3. Comparison of EGF Genotype and Allelic Frequencies in Massachusetts Study Group Patients with Cirrhosis vs Patients With Hepatocellular Carcinoma and Cirrhosis**

<table>
<thead>
<tr>
<th></th>
<th>Cirrhosis and HCC (n = 59)</th>
<th>Odds Ratio (95% Confidence Interval)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/A</td>
<td>9 (15)</td>
<td>1 [Reference]</td>
<td></td>
</tr>
<tr>
<td>A/G</td>
<td>27 (46)</td>
<td>2.4 (1.0-5.4)</td>
<td>.05</td>
</tr>
<tr>
<td>G/G</td>
<td>23 (39)</td>
<td>4.0 (1.6-9.6)</td>
<td>.002</td>
</tr>
<tr>
<td>A</td>
<td>45 (88)</td>
<td>1 [Reference]</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>73 (62)</td>
<td>2.1 (1.4-3.3)</td>
<td>.001</td>
</tr>
</tbody>
</table>

Abbreviation: HCC, hepatocellular carcinoma.
Table 4. Epidermal Growth Factor (EGF) Protein Expression Level by Genotype in Liver Tissue and Serum, and Phospho-EGF Receptor in Liver Tissue

<table>
<thead>
<tr>
<th>Expression Level by Genotype, Median (Range)</th>
<th>P Valuea</th>
<th>G/G vs A/A</th>
<th>Overallb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue EGF, pg/mL</td>
<td>A/A (n = 12)</td>
<td>A/G (n = 12)</td>
<td>G/G (n = 12)</td>
</tr>
<tr>
<td></td>
<td>59 (37-88)</td>
<td>96 (94-108)</td>
<td>140 (82-181)</td>
</tr>
<tr>
<td>Serum EGF, pg/mL</td>
<td>778 (433-1011)</td>
<td>1117 (470-1756)</td>
<td>1378 (663-4233)</td>
</tr>
<tr>
<td>Phospho-EGF receptor, pg/mL</td>
<td>109 (70-138)</td>
<td>118 (75-205)</td>
<td>147 (85-418)</td>
</tr>
</tbody>
</table>

Table 5. General Characteristics of French Study Group

<table>
<thead>
<tr>
<th>A/A (n = 40)</th>
<th>A/G (n = 54)</th>
<th>G/G (n = 27)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean, y</td>
<td>53</td>
<td>54</td>
<td>53</td>
</tr>
<tr>
<td>Men, No. (%)</td>
<td>36 (90)</td>
<td>45 (83)</td>
<td>20 (74)</td>
</tr>
<tr>
<td>White, No. (%)</td>
<td>40 (100)</td>
<td>54 (100)</td>
<td>27 (100)</td>
</tr>
<tr>
<td>Cirrhosis due to alcohol, No. (%)</td>
<td>40 (100)</td>
<td>54 (100)</td>
<td>27 (100)</td>
</tr>
<tr>
<td>Child class, No. (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>9 (23)</td>
<td>5 (9)</td>
<td>5 (19)</td>
</tr>
<tr>
<td>B</td>
<td>9 (23)</td>
<td>27 (50)</td>
<td>10 (37)</td>
</tr>
<tr>
<td>C</td>
<td>22 (55)</td>
<td>22 (41)</td>
<td>12 (44)</td>
</tr>
<tr>
<td>Laboratory values, mean (SD)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Albumin, g/L</td>
<td>33 (10)</td>
<td>31 (7)</td>
<td>31 (6)</td>
</tr>
<tr>
<td>Total bilirubin, µmol/L</td>
<td>3.1 (4.2)</td>
<td>2.6 (2.0)</td>
<td>6.0 (4.0)</td>
</tr>
<tr>
<td>Prothrombin time, %</td>
<td>54 (16)</td>
<td>58 (19)</td>
<td>54 (20)</td>
</tr>
<tr>
<td>Platelet, 1000/µL</td>
<td>154 (101)</td>
<td>135 (64)</td>
<td>106 (62)</td>
</tr>
</tbody>
</table>

Table 6. Comparison of Epidermal Growth Factor Genotype and Allelic Frequencies in French Study Group Patients With Cirrhosis vs Patients With Hepatocellular Carcinoma and Cirrhosis.

<table>
<thead>
<tr>
<th>No. (%) of Patients</th>
<th>Cirrhosis (n = 77)</th>
<th>Cirrhosis and HCC (n = 44)</th>
<th>Odds Ratio (95% Confidence Interval)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/A</td>
<td>28 (36)</td>
<td>12 (27)</td>
<td>1 [Reference]</td>
<td>.29</td>
</tr>
<tr>
<td>A/G</td>
<td>37 (48)</td>
<td>17 (39)</td>
<td>1.1 (0.4-2.6)</td>
<td></td>
</tr>
<tr>
<td>G/G</td>
<td>12 (16)</td>
<td>15 (34)</td>
<td>2.9 (1.1-8.1)</td>
<td>.045</td>
</tr>
<tr>
<td>A</td>
<td>93 (60)</td>
<td>41 (47)</td>
<td>1 [Reference]</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>61 (40)</td>
<td>47 (53)</td>
<td>1.7 (1.0-3.0)</td>
<td>&lt;.044</td>
</tr>
</tbody>
</table>

Note: A/A = G/G compared with A/G.

COMMENT

Much effort has been directed toward understanding the role of EGF receptors and their signaling pathways in transformation, tumor progression, and drug response. The present study highlights the important role of a growth factor itself on the initiation of a primary tumor during the responses of the liver to chronic injury. These data extend previous findings in animal models and provide evidence of the importance of EGF in hepatocellular transformation in humans.

The EGF gene single-nucleotide polymorphism allele distribution was examined in this group of 121 white patients with cirrhosis, of whom 44 had hepatocellular carcinoma arising in their cirrhotic livers. Child class was similar in A/A, A/G, and G/G patients (TABLE 5). Total bilirubin, albumin, and prothrombin-time values were similar among the groups. Patients with a G/G genotype had 2.9-fold odds of developing hepatocellular carcinoma compared with A/A patients (TABLE 6). Logistic regression analysis demonstrated that number of copies of G was significantly associated with hepatocellular carcinoma after adjusting for age, sex, and Child class (G/G patients vs A/G plus A/A patients hazard ratio, 4.87; 95% CI, 1.26-18.77; P = .021).
sults of this study strongly suggest that EGF gene single-nucleotide polymorphism analysis and serum EGF measurements may serve as novel markers for risk of hepatocellular carcinoma in patients with cirrhosis. Epidermal growth factor factor up-regulation is a characteristic of cirrhotic liver disease.28 Notably, human hepatocyte transformation to anchorage-independent growth is enhanced by EGF in a dose-dependent fashion (B.C.F. and K.K.T., unpublished data, 2007). Differences in stability of mRNA transcribed from the 2 alleles are important because they lead to increased EGF mRNA expression in G/G cell lines. Observations in cell lines often do not fully recapitulate in vivo processes; however, these mRNA stability experiments are of significance because of the observation that serum and liver EGF levels are greater in G/G vs A/A patients. It is also possible that other factors influence EGF levels (eg, age, ethnicity, diet, medications), and thereby modulate risk for hepatocellular carcinoma. The lack of an association between this EGF gene polymorphism and plasma EGF levels has been reported in a group of patients without cirrhosis.29 However, the major source of EGF in blood is platelets,23 and therefore we performed EGF measurements in serum and in liver tissue, which are presumed to be most relevant to hepatocyte transformation in cirrhosis.

Whether higher EGF levels are associated with a greater risk for developing cirrhosis is not addressed by this study. However, we speculate that higher EGF does not raise the likelihood for development of cirrhosis because the EGF gene single-nucleotide polymorphism allelic distribution in our cirrhosis control group is similar to what was reported about North American normal controls.30,31 And, whether higher EGF levels are associated with a shorter time to development of cirrhosis is not addressed by this study. In theory, it is conceivable that higher EGF levels speed the onset of cirrhosis in alcoholics or hepatitis virus-infected individuals, and thereby increase the odds of hepatocellular carcinoma in G/G patients compared with A/A patients. However, our observation that the severity of cirrhosis did not differ among our A/A, A/G, and G/G patients argues against this possibility.

Recognizing the extremely high frequency of polymorphic changes in the human genome, Rosenthal and Schwartz49 propose several criteria to establish medically useful links between polymorphisms and disease. First, it is essential to show that the change in the gene causes a relevant alteration in the function or level of the gene product. We have demonstrated modulation of EGF levels by the EGF gene polymorphism; moreover, we have demonstrated a mechanism by which EGF levels are regulated.

Second, the number of cases associating an allele with a particular phenotype must be large enough to be convincing. The current study involves 55 individuals with the G/G single-nucleotide polymorphism in the Massachusetts study group, of which 23 (42%) had hepatocellular carcinoma. Using an independent group of cirrhotic patients, we validated the association between EGF gene single-nucleotide polymorphism and hepatocellular carcinoma.

Third, the beneficial and harmful phenotypes being studied must have clear-cut clinical differences. In the current study, there is little equivocating between the presence and absence of hepatocellular carcinoma in these well-studied populations, in which explanted livers were carefully evaluated for the presence of hepatocellular carcinoma.

Fourth, the plausibility of the hypothesis must be convincing. Studies demonstrating enhanced in vitro transformation in the presence of EGF, combined with animal models in which liver-directed EGF overexpression causes hepatocellular carcinoma provide extremely strong support for the linkage between EGF gene polymorphism and hepatocellular carcinoma.56,17 In addition, it has been proposed that the correlation between a particular single-nucleotide polymorphism and a disease should have practical value. Our observations have significant and immediate practical value in their relevance to tailoring of screening strategies for different cirrhotic populations based on their likelihood of developing hepatocellular carcinoma, identification of other modulators of EGF levels, and development of chemoprevention strategies that target EGF or EGF receptor. If a compound were identified that effectively and safely lowers EGF levels and risk for hepatocellular carcinoma, its use for chemoprevention would likely be cost-effective compared with strategies aimed at early detection and treatment of hepatocellular carcinoma.

Schiffer et al31 demonstrated in a rat model in which diethylnitrosamine induces cirrhosis within 12 weeks and subsequent hepatocellular carcinoma at 18 weeks that concurrent treatment with the selective EGF receptor tyrosine kinase inhibitor gefitinib during weeks 12 through 18 significantly reduced the formation of hepatocellular carcinoma nodules. When combined with these preclinical study results, our findings in humans provide rationale for examination of EGF–EGF receptor pathway as a novel target for chemoprevention in humans. Unlike the situation related to the previously published report linking the EGF gene single-nucleotide polymorphism and melanoma,18 the presence of cirrhosis appears to be an important prerequisite. Thus, one could consider a chemoprevention strategy using agents that block the EGF–EGF receptor pathway in a defined population.

The 2 populations involved in this study differ primarily in the etiology of cirrhosis—predominantly hepatitis C virus in the Massachusetts patients and solely alcohol in the French patients. The effect of the number of copies of G differs in the 2 patient populations. In the Massachusetts patients, each added G copy increased the odds of hepatocellular carcinoma, whereas in the French population, two G copies were required to increase the odds of hepatocellular carcinoma. Additional stud-
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studies are required to further clarify the relationship between different etiologies of cirrhosis and likelihood of transformation associated with EGF gene single-nucleotide polymorphism. Because of the current study’s retrospective design and inability to control for severity of underlying liver disease by liver histopathology (e.g., Ishak score), prospective studies examining larger populations of patients with clinicopathological correlation will be of benefit. In addition, a large majority of patients in the Massachusetts group and all of the patients in the French group were white, and thus the application of these observations to ethnic minorities awaits further study.

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