Prognostic Role of Lemur Tyrosine Kinase-3 Germline Polymorphisms in Adjuvant Gastric Cancer in Japan and the United States

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Abstract

Lemur tyrosine kinase-3 (LMTK3) was recently identified as an estrogen receptor (ER)-α modulator related to endocrine therapy resistance, and its polymorphisms rs9989661 (T>C) T/T genotype and rs8108419 (G>A) G/G or A/G genotype predicted improved outcomes in breast cancer. Because different predominant ER distributions link to breast and gastric cancer and little is known of the prognostic role of LMTK3 in gastric cancer, this study was carried out to clarify the prognostic role of these polymorphisms in gastric cancer. One-hundred and sixty-nine Japanese and 137 U.S. patients with localized gastric adenocarcinoma were enrolled. Genomic DNA was extracted from blood or tissue, and all samples were analyzed by PCR-based direct DNA sequencing. Overall, these polymorphisms were not associated with survival in both cohorts. When gender was considered, in multivariate analysis, harboring rs9989661 T/T genotype was associated with disease-free survival [HR, 4.37; 95% confidence interval (CI), 2.08–9.18; P < 0.0001] and overall survival (OS; HR, 3.69; 95% CI, 1.65–8.24; P = 0.0014) in the Japanese males and time to recurrence (HR, 7.29; 95% CI, 1.07–49.80; P = 0.043) in the U.S. females. Meanwhile, harboring rs8108419 G/G genotype was associated with OS in the Japanese females (HR, 3.04; 95% CI, 1.08–8.56; P = 0.035) and the U.S. males (HR, 3.39; 95% CI, 1.31–8.80; P = 0.012). The prognostic role of these polymorphisms may be negative in gastric cancer. These findings suggest that the estrogen pathway may play a prognostic role in patients with gastric cancer but this may be dependent on the regional differences both in physiology and genetic alterations of gastric cancer. Mol Cancer Ther; 12(10); 2261–72. ©2013 AACR.

Introduction

Gastric cancer is currently the fourth most common malignancy and second leading cause of cancer-related deaths worldwide, with 939,600 new cases diagnosed annually and 738,000 patients succumbing to this disease (1). The National Cancer Center (Tokyo, Japan) reported that 117,320 cases were diagnosed and 50,597 people died from this disease in Japan in 2007. It is estimated that 133,900 new cases will be diagnosed and 49,400 people will die from this disease each year from 2010 to 2014. On the other hand, the American Cancer Society estimated that 21,320 new cases will be diagnosed and 10,540 people will die from this disease in the United States in 2012 (2, 3).

Gastric cancer is a heterogeneous disease, and there are regional differences in epidemiologic and clinicopathologic features. Despite the overall decline in the incidence of gastric cancer over the past several decades, rates of gastroesophageal junction (GEJ) cancer has increased since the 1970s in Western countries, especially in Caucasian males in the United States (4, 5). Meanwhile, both distal gastric cancer and intestinal-type, characterized by atrophic gastritis with intestinal metaplasia due to Helicobacter pylori infection, are more common in East Asia, Eastern Europe, and Central and South America (6). In addition, specific genetic alterations are associated with clinicopathologic features and prognosis in gastric cancer (7). HER2 overexpression is associated with the intestinal-type and GEJ cancers and EGF receptor (EGFR) overexpression is more

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likely to be found in the intestinal-type (8–10), whereas loss-of-function of E-cadherin and c-MET overexpression are more likely to be found in the diffuse-type (11, 12). These molecular diversities of genetic alternations, which reflect clinicopathologic features based on regional differences, have led to different prognosis with a lack of standard chemotherapeutic strategies across the world for patients with gastric cancer (13–15).

In contrast to breast cancer, accumulated epidemiologic studies have suggested that estrogen may have protective effects against gastrointestinal cancer. Male predominant prevalence in gastrointestinal cancer and better survival of young women in colorectal cancer and esophageal cancer have been shown (1, 16, 17). Postmenopausal hormone replacement therapy (HRT) reduced the incidence of colorectal cancer and gastric cancer (18, 19), and conversely, adjuvant antiestrogen, tamoxifen, therapy for breast cancer increased second primary colorectal cancer and gastric cancer (19–21). Because an incidence of gastric cancer in males is more than 2-fold higher than females and the onset of gastric cancer in males is 10 to 17 years earlier compared with females (22, 23), estrogen may critically influence gastric cancer incidence, development, and progression. This epidemiologic evidence suggests a protective estrogen effect against development of gastric cancer; however, its mechanism remains to be elucidated. The effects of estrogen are typically mediated by estrogen receptors (ER): ER-α and -β. In contrast to the reproductive system where ER-α is predominantly expressed, ER-β is the predominant ER expressed in the gastrointestinal tract (24, 25). Expression rates of ER-α and -β in gastric cancer by immunohistochemical staining are 0% to 36% and 11% to 100%, respectively, whereas expression rates of ER-α and -β in corresponding normal stomach tissue are 0% to 25.6% and 34% to 100%, respectively (26–30). Although ER-α is well known to promote growth and is associated with aggressive forms of breast cancer, recent biologic evidence suggests that ER-β has a suppressive effect against ER-α. It was shown that ER-β signaling upregulates integrin gene expression and downregulates several genes in vitro, including IL-6, cyclin D1, VEGF, and Bcl-2, suggesting that ER-β signaling may play a critical role in regulating the inflammatory reaction, proliferation, migration, angiogenesis, and apoptosis (31–34). In addition, loss of ER-β expression in gastric cancer was associated with diffuse-type histology, advanced stage, peritoneal invasion, and worse prognosis (28–30). These molecular biologic data further support the protective role of estrogen via ER-β pathway in gastric cancer.

Lemur tyrosine kinase-3 (LMTK3) belongs to the family of serine–threonine–tyrosine kinases and was recently identified as a regulatory target associated with endocrine therapy resistance in adjuvant breast cancer (35, 36). LMTK3 phosphorlylates and protects ER-α from proteosomal degradation; consequently, leading to ER-α stabilization and activation. Lower LMTK3 protein expression and its germline polymorphisms rs9989661 (T>C) T/T genotype and rs8108419 (G>A) G/G or A/G genotypes were associated with favorable clinicopathologic profiles and better prognosis in ER-αþ breast cancer (35, 37). Meanwhile, we recently reported inverse results that rs9989661 T/T genotype was associated with worse prognosis in colorectal cancer (38, 39). Given these opposing results, which reflect the different biology based on predominant ERs distributions among breast and colorectal cancer and growing data suggesting an important role of estrogen in gastric cancer, we hypothesized that LMTK3 polymorphisms may have different prognostic roles in breast and gastrointestinal cancers. Because the prognostic role of LMTK3 polymorphisms in gastric cancer is unknown and the regional differences in epidemiologic and clinicopathologic features are recognized, we tested whether LMTK3 polymorphisms in gastric cancer will be associated with outcome in two ethnically and epidemiologically different cohorts from Japan and the United States.

Materials and Methods

Patients

Of note, 169 Japanese and 137 U.S. patients with histopathologically confirmed localized (stage I–IV) gastric adenocarcinoma were enrolled from Japan [Fukushima Red Cross Hospital (Fukushima, Japan) and Kitasato University East Hospital (Sagamihara, Japan)], and the United States [University of Southern California/Norris Comprehensive Cancer Center (Los Angeles, CA), Los Angeles County Hospital (Los Angeles, CA), and Memorial Sloan-Kettering Cancer Center (New York, NY)], respectively, between 1991 and 2010. Japanese patients with gastric cancer were treated with D2 lymphadenectomy-based surgery alone or surgery plus 5-FU or fluoropyrimidine-based adjuvant chemotherapy without radiotherapy, whereas U.S. patients were treated with D1–based surgery alone or surgery plus fluoropyrimidine-based adjuvant (radio)-chemotherapy. Patients were followed clinically every 3 months for the first 2 years and then every 6 months. Pathologic stage was decided according to tumor–node–metastasis (TNM) classification sixth edition in both cohorts. This study was approved by the Institutional Review Boards of each institute, and all patients signed an informed consent for the analysis of molecular correlates. This study was carried out adhering to the REPorting recommendations for tumor MARKer prognostic studies (REMARK; ref. 40).

LMTK3 rs9989661 and rs8108419 genotyping

Genomic DNA was extracted from peripheral blood or formalin-fixed paraffin-embedded (FFPE) tissue derived from tumor samples using the QIAmp Kit (Qiagen) according to the manufacturer’s protocol. Extracted DNA was amplified using the following primer set: forward: 5′-GGG ACT TCC CAA GTG GTT-3′ and reverse: 5′-ATC CAA GCC TGG GGT GAG-3′ for rs9989661; forward: 5′-GAG GAC GAG CTT AGA ATC CA-3′ and reverse:
5′-GTT GGT GTG AAC CAG AGC AG-3′ for rs8108419. All samples were analyzed by means of PCR-based direct DNA sequencing. For quality control purposes, a random selection of 10% of the samples was examined for each polymorphism and genotype concordance rate was 100%.

**Immunohistochemistry**
Seventeen (n = 17) of FFPE adjuvant gastric cancer samples from the U.S. cohort were subjected to immunohistochemistry (IHC) to detect LMTK3 protein expression. IHC was conducted at the University of Southern California Immunohistochemistry Clinical Laboratory using the LMTK3 monoclonal antibody (I-17; Santa Cruz Biotechnology, Inc.) at a concentration of 2 μg on full-face excisional tissue sections as previously described (35, 37). Slides were cut at 4-μm thick FFPE adjuvant gastric cancer samples. Negative controls were carried out by omission of the primary antibody. Positive controls were carried out with breast cancer samples (n = 5). LMTK3 immunoreactivity was detected in the nucleus and in the cytoplasm to a variable degree in gastric cancer samples by digital imaging (Leica ICC50) at magnification (×200) with a computer-based interface (Leica Acquire 1.0). Protein expression levels of LMTK3 were determined according to the previous reports (35, 37).

**Statistical analysis**
The primary endpoints were disease-free survival (DFS) in the Japanese cohort and time to recurrence (TTR) in the U.S. cohort, and the secondary endpoint was overall survival (OS) in both cohorts. DFS was defined between the date of surgery and first documented recurrence or death from any cause, whereas TTR was defined between the date of diagnosis and first documented recurrence. OS in the Japanese cohort was defined between the date of surgery and death from any cause, whereas OS in the U.S. cohort was defined between the date of diagnosis and death from any cause. If patients did not meet any endpoints until April 20, 2011, they were censored at the time of last contact. Allelic distribution of the polymorphisms by ethnicity was tested for deviation from Hardy–Weinberg equilibrium using a χ² test with 1 degree of freedom. Linkage disequilibrium between two polymorphisms was assessed using D' and r² values. To evaluate the prognostic value of these polymorphisms, endpoints were estimated using Kaplan–Meier methods and compared by the log-rank test. The Cox proportional hazards regression model with stratification factors were fitted to reevaluate the association between LMTK3 polymorphisms and outcomes considering the imbalance in the distributions of baseline characters in both cohorts. The baseline demographic and clinical markers that remained significantly associated with endpoints in the multivariate analyses (P < 0.1) were included in the final model. With 169 patients in the Japanese cohort and 137 patients in the U.S. cohort, we would have 80% power to detect a minimum HR of 1.8 to 2.0 in DFS and TTR across a range of the variant allele frequencies (0.2–0.5) in a dominant model using a 0.05 level two-sided log-rank test. For a recessive model, a minimum HR is less than 3.8 when the variant allele frequency is 0.2 and approaches 1.8 to 2.0 when the allele frequency is 0.5. The level of significance was set to P < 0.05, and all statistical tests were two-sided and conducted using the SAS statistical package version 9.2 (SAS Institute).

**Results**
A total of 306 patients, 169 Japanese and 137 U.S. patients, with localized gastric cancer were enrolled in this study. The clinicopathologic characteristics and outcomes in both cohorts were summarized in Tables 1 and 2. The clinicopathologic baselines of both cohorts varied considerably. Briefly, the U.S. cohort was more likely to have young, advanced stage, poorly differentiated pathology and worse general condition compared with the Japanese cohort. With respect to primary tumor site, significantly higher incidence of GEJ cancer in males was found in the U.S. cohort (Supplementary Table S1). Furthermore, adjuvant treatments of both cohorts were lacking of unity. The median follow-up periods were 4.0 years in the Japanese cohort and 3.3 years in the U.S. cohort, respectively. The median DFS and OS in the Japanese cohort were 4.8 and 5.8 years, whereas the median TTR and OS in the U.S. cohort were 2.8 and 4.7 years, respectively. All patients in the Japanese cohort and 131 patients (96%) in the U.S. cohort were followed until death or the end of the study period. One hundred and fifty-five patients (90%) in the Japanese cohort and 127 patients (93%) in the U.S. cohort complied with the follow-up schedule. Age, stage, T-category, N-category, performance status, and adjuvant chemotherapy were significantly associated with DFS in the Japanese cohort; on the other hand, stage, T-category, N-category, tumor site, and adjuvant chemotherapy were also significantly associated with TTR in the U.S. cohort.

**LMTK3 rs9989661 and rs8108419 genotyping**
Final success rates of LMTK3 genotyping in the Japanese and the U.S. cohorts were 167 (99%) and 125 (91%) for rs9989661 and 165 (98%) and 127 (93%) for rs8108419, respectively. The allelic frequencies of rs9989661 were not within the probability limits of Hardy–Weinberg equilibrium in both cohorts (χ² test; P < 0.05). In addition, significantly different allelic distributions in LMTK3 rs9989661 were found between both cohorts (Supplementary Table S2). Moreover, strong linkage disequilibrium between rs9989661 C allele and rs8108419 G allele was found in the Japanese cohort (D' = 0.967; r² = 0.338), whereas weak linkage disequilibrium between rs9989661 T allele and rs8108419 G allele was found in the U.S. cohort (D' = 0.512; r² = 0.042). There were no significant differences between genotypes of these polymorphisms and clinical characteristics including differentiation, Lauren classification, and tumor site in both cohorts (all χ² test; P > 0.05; data were not shown).
Univariate analysis for LMTK3 polymorphisms

In both polymorphisms, no significant differences of endpoints in overall patients were found in both cohorts (Supplementary Table S3). After analyzing according to gender, in rs9989661, the Japanese males harboring T/T genotype had shorter median DFS of 0.9 years [95% confidence interval (CI), 0.3–6.1 years) compared with median DFS of 7.0 years (95% CI, 6.6–7.3 years) among the female group. In both polymorphisms, no significant differences of DFS and OS were observed among the T/C genotype group. In the Japanese cohort, the median DFS and OS of both polymorphisms did not differ significantly between the T/T and T/C genotypes (Table 3). The U.S. females harboring T/T genotype had a shorter median TTR of 1.7 years (95% CI, 0.7–7.0 years) compared with 7.0 years

### Table 1. Japanese cohort characteristics and clinical outcome: DFS and OS

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>DFS</th>
<th>OS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median DFS, y (95% CI)</td>
<td>HR (95% CI)</td>
</tr>
<tr>
<td>Age, y</td>
<td>20.1&lt;sup&gt;b&lt;/sup&gt; (4.8–20.1&lt;sup&gt;b&lt;/sup&gt;)</td>
<td>1 (Reference)</td>
</tr>
<tr>
<td></td>
<td>Age &lt;65</td>
<td>65 (38%)</td>
</tr>
<tr>
<td></td>
<td>Age 65–74</td>
<td>60 (36%)</td>
</tr>
<tr>
<td></td>
<td>Age ≥75</td>
<td>44 (26%)</td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>109 (64%)</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>60 (36%)</td>
</tr>
<tr>
<td>Stage</td>
<td>IB</td>
<td>28 (16%)</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>53 (31%)</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>60 (36%)</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>28 (16%)</td>
</tr>
<tr>
<td>Tumor stage</td>
<td>T1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>12 (7%)</td>
</tr>
<tr>
<td></td>
<td>T2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>66 (39%)</td>
</tr>
<tr>
<td></td>
<td>T3&lt;sup&gt;e&lt;/sup&gt;</td>
<td>88 (52%)</td>
</tr>
<tr>
<td></td>
<td>T4&lt;sup&gt;e&lt;/sup&gt;</td>
<td>3 (2%)</td>
</tr>
<tr>
<td>N</td>
<td>N0</td>
<td>36 (21%)</td>
</tr>
<tr>
<td></td>
<td>N1</td>
<td>85 (50%)</td>
</tr>
<tr>
<td></td>
<td>N2</td>
<td>33 (20%)</td>
</tr>
<tr>
<td></td>
<td>N3</td>
<td>15 (9%)</td>
</tr>
<tr>
<td>Tumor site</td>
<td>Lower</td>
<td>55 (32%)</td>
</tr>
<tr>
<td></td>
<td>Middle</td>
<td>59 (35%)</td>
</tr>
<tr>
<td></td>
<td>Upper</td>
<td>49 (29%)</td>
</tr>
<tr>
<td></td>
<td>GEJ</td>
<td>3 (2%)</td>
</tr>
<tr>
<td></td>
<td>UML</td>
<td>3 (2%)</td>
</tr>
<tr>
<td>Tumor differentiation</td>
<td>Well–moderate</td>
<td>68 (40%)</td>
</tr>
<tr>
<td></td>
<td>Poor</td>
<td>101 (60%)</td>
</tr>
<tr>
<td>PS</td>
<td>ECOG 0</td>
<td>157 (93%)</td>
</tr>
<tr>
<td></td>
<td>ECOG 1</td>
<td>12 (7%)</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>No</td>
<td>60 (36%)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>109 (64%)</td>
</tr>
</tbody>
</table>

Abbreviations: ECOG, Eastern Cooperative Oncology Group; PS, performance status; UML, upper-middle–lower.

<sup>a</sup>On the basis of log-rank test.
<sup>b</sup>Estimates were not reached.
<sup>c</sup>No events occurred and estimates were not obtained.
<sup>d</sup>Grouped together for the estimates of HR.
### Table 2. The U.S. cohort characteristics and clinical outcome: TTR and OS

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>n (%)</th>
<th>Median TTR, y (95% CI)</th>
<th>HR (95% CI)</th>
<th>P*</th>
<th>Median OS, y (95% CI)</th>
<th>HR (95% CI)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>&lt;60</td>
<td>80 (58%)</td>
<td>2.2 (1.5–14.5)</td>
<td>1</td>
<td>0.42</td>
<td>4.7 (3.8–14.6)</td>
<td>1</td>
<td>0.65</td>
</tr>
<tr>
<td>≥60</td>
<td>57 (42%)</td>
<td>3.7 (2.1–12.3)</td>
<td>0.81 (0.48–1.36)</td>
<td>0.68</td>
<td>4.5 (3.3–7.3)</td>
<td>1.14 (0.64–2.05)</td>
<td>0.32</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>83 (61%)</td>
<td>2.3 (1.8–7.0)</td>
<td>1</td>
<td>0.85</td>
<td>4.1 (3.3–7.3)</td>
<td>1</td>
<td>0.32</td>
</tr>
<tr>
<td>Female</td>
<td>54 (39%)</td>
<td>7.0 (1.5–8.3)</td>
<td>0.95 (0.56–1.63)</td>
<td>0.92</td>
<td>7.3 (3.8–8.3)</td>
<td>0.72 (0.37–1.39)</td>
<td>0.32</td>
</tr>
<tr>
<td>Race</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>White</td>
<td>63 (46%)</td>
<td>1.7 (1.2–4.4)</td>
<td>1</td>
<td>0.085</td>
<td>3.8 (2.7–5.5)</td>
<td>1</td>
<td>0.040</td>
</tr>
<tr>
<td>African American</td>
<td>1 (1%)</td>
<td>0.5</td>
<td>c</td>
<td>0.5b</td>
<td>0.5</td>
<td>c</td>
<td>0.5b</td>
</tr>
<tr>
<td>Asian</td>
<td>28 (20%)</td>
<td>7.0 (2.3–14.5)</td>
<td>0.45 (0.23–0.91)</td>
<td>0.15</td>
<td>7.3 (3.3–14.6)</td>
<td>0.45 (0.20–1.03)</td>
<td>0.32</td>
</tr>
<tr>
<td>Hispanic</td>
<td>45 (33%)</td>
<td>3.7 (2.1–10.7)</td>
<td>0.63 (0.34–1.17)</td>
<td>0.030</td>
<td>10.7 (3.6–10.7)</td>
<td>0.36 (0.15–0.85)</td>
<td>0.32</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>IB</td>
<td>12 (8%)</td>
<td>4.3 (2.2–4.3)</td>
<td>1</td>
<td>0.013</td>
<td>4.4</td>
<td>c</td>
<td>0.013</td>
</tr>
<tr>
<td>II</td>
<td>36 (26%)</td>
<td>7.0 (2.9–10.7)</td>
<td>1.56 (0.53–6.98)</td>
<td>0.85</td>
<td>5.4 (1.6–10.7)</td>
<td>1</td>
<td>0.85</td>
</tr>
<tr>
<td>III</td>
<td>71 (52%)</td>
<td>1.8 (1.4–2.8)</td>
<td>3.24 (0.78–13.5)</td>
<td>0.19</td>
<td>3.8 (2.8–7.3)</td>
<td>1.31 (0.69–2.50)</td>
<td>0.32</td>
</tr>
<tr>
<td>IV</td>
<td>18 (13%)</td>
<td>1.6 (1.2–3.8)</td>
<td>4.00 (0.86–18.5)</td>
<td>0.19</td>
<td>7.3 (1.4–7.3)</td>
<td>1.33 (0.43–4.09)</td>
<td>0.32</td>
</tr>
<tr>
<td>Tumor stage</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>T1</td>
<td>4 (3%)</td>
<td>8.3 (2.9–8.3)</td>
<td>1</td>
<td>0.004</td>
<td>5.4</td>
<td>1</td>
<td>0.32</td>
</tr>
<tr>
<td>T2</td>
<td>44 (32%)</td>
<td>8.3 (2.9–8.3)</td>
<td>1</td>
<td>0.004</td>
<td>5.4</td>
<td>1</td>
<td>0.32</td>
</tr>
<tr>
<td>T3</td>
<td>79 (58%)</td>
<td>1.7 (1.4–4.4)</td>
<td>2.04 (1.14–3.67)</td>
<td>0.20</td>
<td>4.5 (3.3–7.3)</td>
<td>1.40 (0.73–2.68)</td>
<td>0.20</td>
</tr>
<tr>
<td>T4</td>
<td>10 (7%)</td>
<td>7.0 (2.3–14.5)</td>
<td>0.65 (0.36–1.19)</td>
<td>0.20</td>
<td>7.3 (3.8–14.6)</td>
<td>0.59 (0.31–1.13)</td>
<td>0.20</td>
</tr>
<tr>
<td>Lauren</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Diffuse</td>
<td>40 (29%)</td>
<td>3.7 (1.8–8.9)</td>
<td>1</td>
<td>0.87</td>
<td>7.3</td>
<td>1</td>
<td>0.74</td>
</tr>
<tr>
<td>Intestinal</td>
<td>50 (36%)</td>
<td>7.0 (2.1–14.5)</td>
<td>0.87 (0.45–1.67)</td>
<td>0.001</td>
<td>5.7</td>
<td>1.10 (0.48–2.51)</td>
<td>0.001</td>
</tr>
<tr>
<td>Mixed</td>
<td>21 (15%)</td>
<td>12.3 (7.1–12.3)</td>
<td>1.04 (0.45–2.41)</td>
<td>0.004</td>
<td>3.6</td>
<td>1.52 (0.51–4.58)</td>
<td>0.004</td>
</tr>
<tr>
<td>Tumor site</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stomach</td>
<td>88 (64%)</td>
<td>4.4 (2.1–14.5)</td>
<td>1 (Reference)</td>
<td>0.030</td>
<td>7.3</td>
<td>1 (Reference)</td>
<td>0.030</td>
</tr>
<tr>
<td>GEJ</td>
<td>38 (28%)</td>
<td>1.6 (1.1–2.9)</td>
<td>1.97 (1.15–3.37)</td>
<td>0.030</td>
<td>3.4</td>
<td>2.50 (1.35–4.60)</td>
<td>0.030</td>
</tr>
<tr>
<td>Unknown</td>
<td>11 (8%)</td>
<td>4.4 (3.2–4.2)</td>
<td>0.77 (0.24–2.54)</td>
<td>0.030</td>
<td>4.4</td>
<td>3.3–4.4)</td>
<td>0.51 (0.07–3.89)</td>
</tr>
<tr>
<td>Type of chemotherapy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-FU/LV</td>
<td>70 (51%)</td>
<td>7.0 (2.8–10.6)</td>
<td>1</td>
<td>&lt;0.001</td>
<td>7.3</td>
<td>1</td>
<td>0.009</td>
</tr>
<tr>
<td>S-FU/LV/oxaliplatin</td>
<td>19 (14%)</td>
<td>1.6 (1.1–2.9)</td>
<td>2.65 (1.22–5.75)</td>
<td>0.001</td>
<td>2.4</td>
<td>4.07 (1.62–10.19)</td>
<td>0.001</td>
</tr>
<tr>
<td>5-FU, CDDP, CPT-11</td>
<td>22 (17%)</td>
<td>14.5 (1.2–14.5)</td>
<td>1.25 (0.58–2.69)</td>
<td>0.001</td>
<td>4.1</td>
<td>1.32 (0.57–3.05)</td>
<td>0.001</td>
</tr>
<tr>
<td>Radiation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>88 (64%)</td>
<td>2.5 (1.8–14.5)</td>
<td>1</td>
<td>0.92</td>
<td>4.5</td>
<td>1</td>
<td>0.68</td>
</tr>
<tr>
<td>No</td>
<td>48 (35%)</td>
<td>3.7 (1.7–12.3)</td>
<td>1.03 (0.60–1.76)</td>
<td>0.92</td>
<td>5.4</td>
<td>3.8–12.3)</td>
<td>0.89 (0.48–1.63)</td>
</tr>
</tbody>
</table>

Abbreviations: CDDP, cisplatin; ECOG, Eastern Cooperative Oncology Group; S-FU, fluorouracil; LV, Leucovorin; PS, performance status.

aOn the basis of log-rank test.

bEstimates were not reached.

cNo events occurred and estimates were not obtained.

d,eGrouped together for the estimates of HR.
Table 3. *LMTK3* polymorphisms and survival in Japanese cohort by gender

<table>
<thead>
<tr>
<th>LMTK3 Polymorphisms and DFS and OS in Japanese male patients with gastric cancer</th>
<th>DFS</th>
<th>OS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LMTK3 rs8108419</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/A <em>a</em></td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>A/G <em>a</em></td>
<td>26</td>
<td>2.3 (1.2–20.1)</td>
</tr>
<tr>
<td>G/G</td>
<td>78</td>
<td>4.8 (2.0–15.2)</td>
</tr>
<tr>
<td><em>P</em></td>
<td>0.62</td>
<td>0.46</td>
</tr>
<tr>
<td><strong>LMTK3 rs9989661</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C/C <em>a</em></td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>C/T</td>
<td>37</td>
<td>20.1 (2.4–20.1)</td>
</tr>
<tr>
<td><em>P</em></td>
<td>0.030</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>LMTK3 rs8108419</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/A <em>a</em></td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>A/G</td>
<td>17</td>
<td>2.5 (1.6–16.1)</td>
</tr>
<tr>
<td>G/G</td>
<td>36</td>
<td>15.4 (1.9–15.4)</td>
</tr>
<tr>
<td><em>P</em></td>
<td>0.76</td>
<td>0.13</td>
</tr>
<tr>
<td><strong>LMTK3 rs9989661</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C/C <em>a</em></td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>C/T</td>
<td>27</td>
<td>15.4 (1.9–15.4)</td>
</tr>
<tr>
<td><em>P</em></td>
<td>0.79</td>
<td>0.86</td>
</tr>
</tbody>
</table>

*Combined in the analysis in the dominant or recessive model.
*On the basis of the log-rank test in the univariate analysis and Wald test in the multivariate analysis within Cox regression model.
*Estimates were not reached.
*Adjusted for stage (I, II, III, and IV as categorical), age (<65, 65–74, ≥75 years as ordinal), and type of adjuvant therapy (no vs. yes).

(95% CI, 3.7–8.3 years) for CT or CC genotype (HR, 2.70; 95% CI, 1.01–7.19; *P* = 0.025), however, no significant difference was found in OS (Table 4). With respect to the U.S. males and the Japanese females in univariate analysis, no significant differences were found in DFS, TTR, and OS in terms of both polymorphisms (Tables 3 and 4). On the other hand, even upon considering gender, rs8108419 showed no significant differences in the endpoints in both cohorts.

**Multivariate analysis for LMTK3 polymorphisms**

Multivariate analysis for *LMTK3* rs9989661 and rs8108419 was stratified by age, stage, and adjuvant chemotherapy in the Japanese cohort and by N stage, race, and adjuvant chemotherapy in the U.S. cohort. *LMTK3* rs9989661 remained significantly associated with DFS (HR, 4.37; 95% CI, 2.08–9.18; *P* < 0.0001) and OS (HR, 3.69; 95% CI, 1.65–8.24; *P* = 0.0014) in the Japanese males and TTR (HR, 7.29; 95% CI, 1.07–49.80; *P* = 0.043) in the U.S. females (Tables 3 and 4). *LMTK3* rs8108419 was also associated with only OS in the Japanese females (HR, 3.04; 95% CI, 1.08–8.56; *P* = 0.035) and in the U.S. males (HR, 3.39; 95% CI, 1.31–8.80; *P* = 0.012; Tables 3 and 4).

**Immunohistochemistry**

In addition to detection of *LMTK3* polymorphisms, we conducted IHC to detect *LMTK3* protein expression in gastric cancer tissue. All 17 samples were stained positive for *LMTK3* protein expression, with 64.7% (n = 11) staining cytoplasmic only, 11.7% (n = 2) nuclear only, and 23.5% (n = 4) staining positive for both nuclear and cytoplasmic (Fig. 1).

**Discussion**

Accumulated epidemiologic data suggest that female have an advantage in gastrointestinal cancer with a lower incidence of gastrointestinal cancer reported in females and longer survival in young women particular in colorectal cancer and esophageal cancer have been shown (1, 16, 17). Moreover, postmenopausal HRT reduced the incidence of colorectal cancer and gastric cancer (18, 19). Because of the significant lower incidence and delayed...
onset in females compared with males, gastric cancer may be strongly affected by estrogen (22, 23). This epidemiologic evidence strongly suggests the protective role of estrogen in gastric cancer. This protective estrogen effect in gastric cancer should be affected by plasma estradiol (E2) levels and ERs expression in stomach. Plasma E2 levels in postmenopausal women can be affected by estrogen (22, 23). This epidemiologic evidence strongly suggests the protective role of estrogen in gastric cancer.

**Table 4. LMTK3 polymorphisms and survival in the U.S. cohort by gender**

| LMTK3 polymorphisms and TTR OS in the U.S. male patients with gastric cancer |  |  |
| LMTK3 rs8108419 |  |  |
| A/A | A/G | G/G |
| A/G | 23 | 2.5 (1.8–14.5) | 1 (Reference) | 1 (Reference) | 5.7 (2.8–14.6) | 1 (Reference) | 1 (Reference) |
| G/G | 48 | 2.3 (1.5–7.0) | 1.48 (0.74–2.95) | 1.70 (0.76–3.78) | 3.4 (2.8–5.5) | 1.68 (0.79–3.57) | 3.39 (1.31–8.80) |
| P | 0.24 | 0.20 | 0.17 | 0.012 |

| LMTK3 rs9989661 |  |  |
| C/C | C/T | T/T |
| C/T | 15 | 2.5 (2.1–7.0) | 1.04 (0.52–2.09) | 1.09 (0.35–3.38) | 4.5 (2.8–14.6) | 1 (Reference) | 1 (Reference) |
| T/T | 45 | 2.1 (1.5–10.7) | 0.90 | 0.88 | 0.77 | 0.96 |

| LMTK3 polymorphisms and TTR OS in the U.S. female patients with gastric cancer |  |  |
| LMTK3 rs8108419 |  |  |
| A/A | A/G | G/G |
| A/G | 15 | 7.0 (1.5–7.0) | 1.17 (0.42–3.26) | 0.75 (0.15–3.76) | 7.3 (1.9–7.3) | 1 (Reference) | 1 (Reference) |
| G/G | 30 | 3.7 (1.7–8.3) | 0.76 | 0.72 | 5.4 (3.8–8.3) | 1.41 (0.36–5.49) | 5.81 (0.46–74.00) |
| P | 0.18 |

| LMTK3 rs9989661 |  |  |
| C/C | C/T | T/T |
| C/T | 16 | 7.0 (3.7–8.3) | 1 (Reference) | 1 (Reference) | 7.3 (3.8–8.3) | 1 (Reference) | 1 (Reference) |
| T/T | 28 | 1.7 (0.7–7.0) | 2.70 (1.01–7.19) | 7.29 (1.07–49.80) | 4.5 (2.2–7.0) | 1.47 (0.42–5.13) | 1.27 (0.08–20.38) |
| P | 0.052 | 0.043 |

| aCombined in the analysis in the dominant or recessive model. |
| bOn the basis of the log-rank test in the univariate analysis and Wald test in the multivariate analysis within Cox regression model. |
| cEstimates were not reached. |
| dAdjusted for N stage (two groups: N0, N1 vs. N2, N3) and stratified by race (four groups: White, African American, Asian, and Hispanic) and adjuvant therapy (four groups: 5-FU/LV; 5-FU/LV/oxaliplatin; 5-FU, CDDP, CPT-11; none). |
are consistent with previous colorectal cancer results. These opposite results between breast and gastrointestinal cancers in LMTK3 polymorphisms are consistent with the different ER distributions in the breast and in the gastrointestinal tract (24, 25), suggesting that the prognostic role of LMTK3 polymorphisms reflects different predominant ER distributions in different organs. LMTK3 enhances the estrogen pathway at least partly via ER-α phosphorylation (35–37); however, few data are available in terms of a regulation of ER-β phosphorylation. Extracellular signal–regulated kinase (ERK) 1/2 phosphorylated both ER-α and -β, indicating that one kinase is possibly responsible for phosphorylation of both ERs (46, 47). Moreover, it was reported that phosphorylation of ER-β was associated with better survival in ER-α+ breast cancer via posttranslational modifications (48). These data may suggest the hypothesis that in gastrointestinal, LMTK3 could phosphorylate ER-β, resulting in its stabilization and activation in the same manner as ER-α (35–37); consequently, leading to better survival by enhancing ER-β pathway (Fig. 2). The functions of these polymorphisms remain unclear; hence, the functional single-nucleotide polymorphism (F-SNP) database was used for the LMTK3 rs9989661 and rs8108419. F-SNP predicted changes in transcriptional regulation for both polymorphisms (49). Because these polymorphisms are an intronic germline polymorphism, it is possible for these variations to impact transcription factor–binding sites. These findings suggest that these polymorphisms may affect LMTK3 gene expression levels (50). Besides polymorphic results, we

Figure 1. Immunohistochemical staining of LMTK3 with the anti-LMTK3 mouse monoclonal antibody (Santa Cruz Biotechnology). Specimens were processed as described in Materials and Methods. A, LMTK3 protein expression in breast cancer samples were used as positive controls (>200; Leica ICC50). Negative controls were carried out by omission of the primary antibody (not shown). B, LMTK3 protein expression in adjuvant gastric cancer samples from USC cohort (>200; Leica ICC50).
confirmed that \textit{LMTK3} protein was highly expressed in gastric cancer tissue with variable staining patterns. To our knowledge, this is the first evidence that \textit{LMTK3} protein exists in gastrointestinal tract. These results indicate not only the rationale of this study but also the possibility that \textit{LMTK3} protein expression levels might be useful to predict outcome in gastric cancer. Further molecular and biologic analysis \textit{in vitro} and \textit{in vivo} are ongoing including the study of the functional role of these SNPs.

In this study, rs9989661 T/T genotype and rs8108419 G/G genotype were associated with worse outcome, especially rs9989661 T/T genotype predicted recurrence of gastric cancer; hence, rs9989661 may reflect more cancer-specific prognosis in gastric cancer. On the other hand, rs8108419 G/G genotype was not significantly associated with OS in the univariate analysis; however, it became significant in the multivariate analysis in Japanese females and U.S. males. Because Japanese females carrying the G allele are more likely to be younger than those carrying the A allele and younger Japanese patients showed a longer OS, this genotype would not be significantly associated with OS in the univariate analysis. The multivariate analysis of rs8108419 revealed the true association with OS when adjusting the imbalance in the distribution of the baseline prognostic factors; hence, rs8108419 G/G genotype might serve as negative prognostic factors in OS.

However, this association in rs9989661 was found only in the Japanese males and the U.S. females. Postmenopausal Japanese women have the lowest E2 levels among subgroups in this study, according to previous reports (Supplementary Table S4; refs. 44, 45). Lower incidence of postmenopausal breast cancer was found in Japanese females compared with U.S. females (51), and there is a relatively small frequency of HRT in Japan compared with the Western countries (<10% and >40%, respectively), according to case–control studies (51, 52). Furthermore, HRT increased postmenopausal breast cancer in the Western countries, whereas this evidence was not shown to be significant in Japan (53). This evidence is consistent with lower estrogen levels of postmenopausal women in Japan compared with those of the United states and supports that postmenopausal Japanese females have less estrogen effect; therefore, Japanese females with this genetic variation may not show a positive impact. Meanwhile, despite the highest E2 level, the U.S. males were not impacted either. This may in part be explained by the fact that the U.S. males had the highest incidence of GEJ cancer in this study (Supplementary Table S1) and higher rate of HER2 overexpression is expected in GEJ cancers (24%–32%; refs. 8, 9). In ER-positive operable breast cancer treated with adjuvant tamoxifen, HER2 overexpression was associated with poor prognosis (54). Also, HER2-positive metastatic breast cancer is less responsive to any type of endocrine treatment (55). Moreover, increasing data from preclinical studies have shown that cross-talk between LMTK3 and ERs in breast cancer and gastric cancer. ER-α and -β have opposing effects on each other. ER-α is predominantly expressed in breast cancer, whereas ER-β is predominant expression in gastric cancer. LMTK3 phosphorylates ER-α and protects it from proteosomal degradation in breast cancer. It is proposed that LMTK3 may phosphorylate ER-β, in a similar mechanism shown for ER-α in breast cancer, leading to its activation. P, phosphate.
between HER2 and ER leads to a hormone-independent state in HER2 co-overexpression in breast cancer cells. This occurs via the redistribution of ER nuclear to cytoplasmic or upregulation of ERK1/2, probably resulting in the development for the endocrine resistance in ER-positive breast cancer (56). These data suggest that the GEJ cancer may not be or less influenced by estrogen efficacy. An alternative possibility, although there are no specific data, is that the expression rate of ER-β in GEJ cancer may be lower than those of stomach cancer. These diversities based on physiologic and clinicopathologic backgrounds among gender and region may account for gender and regional specific outcomes in this study. On the other hand, rs8108419 G/G genotype was associated with OS without tumor recurrence in the U.S. males and the Japanese females. These results were also opposite to the data in breast cancer results, suggesting different predominant ERs distributions between the breast and the gastrointestinal tract. However, it is not clear why these two polymorphisms were associated with different endpoints, DFS/TTR or OS, in U.S. and Japanese populations. Significant different allele frequencies in rs9989661 in the two cohorts and different linkage disequilibrium, rs9989661 C allele and rs8108419 G allele, in the Japanese cohort and rs9989661 T allele and rs8108419 G allele in the U.S. cohort, may explain these data. In addition, the small number of patient population along with different definitions of endpoints, clinicopathologic baselines, surgical technique of lymphadenectomy, and different adjuvant treatment in the two cohorts might impact outcomes and associations. Further experiments are warranted to elucidate the molecular mechanisms how these polymorphisms exert their biologic effect.

The prognostic role of LMTK3 polymorphisms reflects predominant ER distribution in each organ, and its prognostic impact should be taken into account given the complexities consisting of regional differences both in physiology and genetic alternations of gastric cancer.

There are several limitations in this study. We must recognize that there are differences in standard clinical practices between Japan and the United States. Some clinical information is missing due to the retrospective nature of data collection, leading to different endpoints and different definitions of endpoints. In addition, not all patients completed to follow-up schedules; therefore, potential selection bias should be considered. These issues may keep firm conclusions at a distance; nevertheless, our consistent results between breast and gastrointestinal cancers reflecting predominant ERs may set a precedent for future researches in the new field of gastrointestinal cancer.

Our results in the LMTK3 polymorphisms analysis in gastric cancer are the first in gastrointestinal cancer and shed new light on the differences between the responses on ER-α versus ER-β-expressing cancers; however, several biologic issues remain to be elucidated with the goal of shedding light on the new possibility of prevention and possible treatment in gastrointestinal cancer. Further biologic molecular studies will elucidate these complexities. In conclusion, LMTK3 polymorphisms may serve as a prognostic factor candidate in gastric cancer and may help to select patients who benefit from more careful observation or aggressive treatment. These data suggest that the estrogen pathway may be a novel target for treatment strategy in gastrointestinal cancer. Further functional correlative preclinical analyses and external clinical validation studies are needed to validate these results.

Disclosure of Potential Conflicts of Interest

S. Stintzing has honoraria from speakers bureau of Roche AG, Merck KG, Amgen GmbH, and Sanofi-Aventis and is a consultant/advisory board member for Merck KG. No potential conflicts of interest were disclosed by the other authors.

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Wakatsuki, M.J. LaBonte, S. Stintzing, M. Watanabe, J. Stebbing
Study supervision: M. Shah, H.-J. Lenz

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Prognostic Role of Lemur Tyrosine Kinase-3 Germline Polymorphisms in Adjuvant Gastric Cancer in Japan and the United States

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