Downregulation of LGI1 promotes tumor metastasis in esophageal squamous cell carcinoma

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Here, we report the characterization of a candidate tumor suppressor gene, leucine-rich glioma inactivated 1 (LGI1), in human esophageal squamous cell carcinoma (ESCC). Downregulation of LGI1 has been detected in approximately 50% of primary ESCCs, which was significantly associated with advanced clinical stage (P < 0.001), lymph node metastasis (P < 0.001), tumor invasion (P = 0.009) and poor disease-specific survival (P < 0.001). Functional studies found that LGI1 could inhibit cell growth, clonogenicity, cell motility and tumor formation in nude mice. Mechanistic investigations suggested that LGI1 acted through extracellular signal-regulated kinase (ERK1/2) signaling to downregulate matrix metalloproteinase (MMP)-3 expression and subsequently suppressed tumor metastasis. Taken together, our study revealed that LGI1 plays an important tumor suppressive role in the development and progression of ESCC, with possible application in clinics as a biomarker and a potential new therapeutic target.

Introduction

Esophageal cancer (EC) is among the eighth most common malignancy and the sixth leading cause of cancer-related mortality worldwide, with occurrence rates varying greatly by geographic locations and ethnicity (1,2). Histologically, esophageal squamous cell carcinoma (ESCC), the most prevalent pathological type of EC, predominates in eastern countries, particularly in China, with a proportion of >90% of all EC (3,4). Despite tremendous progress in diagnosis and therapeutic options, the average overall 5 year survival rate for ESCC is approximately 10–41% (5–7). Therefore, it is imperative to identify and characterize more sensitive and specific molecular markers for early detection and therapeutic targets (8,9).

Canter can be regarded as a genetic disease occurring as a result of progressive accumulation of genetic aberrations (10). The etiology of EC is a complex process involving multiple sequential genetic events resulting in activation of oncogenes and inactivation of tumor suppressor genes. The complementary DNA (cDNA) microarray is a powerful tool for identifying the individual genes and pathways involved in the tumorogenesis. Recently, our group exploited an Affymetrix cDNA microarray to compare differentially expressed genes between 10 pairs of ESCC tumors and their adjacent non-tumor tissues. About 220 downregulated genes were identified including leucine-rich glioma inactivated 1 (LGI1). LGI1 gene, located in 10q24, encodes a predicted 557 amino acid polypeptide. LGI1 is a member of the family of extracellular leucine-rich repeat (LRR) proteins, characterized by the conservation of a phenylalanine at position 20 in the LRR (12). The LGI1 gene was originally isolated at the breakpoint of a reciprocal chromosome translocation (t(10;19) (q24;q13) in the T98G glioblastoma cell line (11). Previous studies have shown that LGI1 is frequently inactivated in a number of tumors, including brain tumors, oral squamous cell carcinoma and prostate cancer (11,13–15). To date, the role of LGI1 in ESCC has not been explored.

In this study, we studied LGI1 expression status and its clinical significance in ESCC. Both in vitro and in vivo functional assays were performed to characterize the biological effects of LGI1 in ESCC tumorigenicity and metastasis. The tumor suppressive mechanism of LGI1 was also investigated.

Materials and methods

Cell lines and clinical samples

Six Japanese ESCC cell lines (KYS30, KYSE140, KYSE180, KYSE410, KYSE510 and KYSE520) were acquired from DSMZ (Braunschweig, Germany), the German Resource Centre for Biological Material (16). One Chinese ESCC cell line, HKES1, was kindly provided by Prof. Srivastava (Department of Pathology, The University of Hong Kong), and two Chinese ESCC cell lines, EC18 and EC109, were kindly provided by Prof. Tao (Department of Anatomy, The University of Hong Kong). All nine human ESCC cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum. All cell lines used in this study were regularly authenticated by morphological observation and tested for absence of Mycoplasma contamination (MycopAlert; Lonza Rockland, Rockland, ME). The primary ESCC tumor tissues and their matched non-tumorous tissues from surgical resection were obtained from Linzhou Cancer Hospital (Henan, China). No patients recruited in this study have received neoadjuvant or adjuvant treatment. All clinical samples used in this study were approved by the committee for ethical review of research involving human subjects at Zhengzhou University, Sun Yat-Sen University and the University of Hong Kong.

Quantitative real-time–PCR

Total RNA was extracted from clinical samples and cultured cell lines using TRIzol reagent (Invitrogen), and was reverse transcribed with random primers using an Advantage RT-for-PCR Kit (Clontech Laboratories) according to the manufacturer’s instructions. Quantitative real-time PCR (qRT–PCR) was performed to detect levels of the corresponding glyceraldehyde 3-phosphate dehydrogenase (GAPDH), LGI1 and several matrix metalloproteinases (MMPs) using a SYBR Green PCR Kit (Applied Biosystems) and an ABI 7900HT Fast Real-time PCR System (Applied Biosystems, Foster City, CA). The GAPDH was used as an internal control for each specific gene. The relative levels of expression were quantified and analyzed by using SDS 2.3 software (Applied Biosystems). The real-time value for each sample was averaged and compared using the Ct method. The relative expression level (defined as fold change) of target gene (2−ΔΔCt) was normalized to the endogenous GAPDH reference (AC1) and related to the amount of target gene in control sample, which was defined as the calibrator at 1.0. Three independent experiments were performed to analyze the relative gene expression and each sample was tested in triplicate. Supplementary Table S1, available at Carcinogenesis Online, provides a list of the primer sequence used in the qRT–PCR assay.

Abbreviations: cDNA, complementary DNA; DSS, disease-specific survival; EC, esophageal cancer; ERK1/2, extracellular signal-regulated kinase; ESCC, esophageal squamous cell carcinoma; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IHC, immunohistochemical; LGI1, leucine-rich glioma inactivated 1; LRR, leucine-rich repeat; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; mRNA, messenger RNA; NTC, non-target control; PMA, phorbol myristate acetate; qRT–PCR, quantitative real-time–PCR; shRNA, small hairpin RNA; TMA, tissue microarray.

These authors contributed equally to this work.
Fig. 1. Downregulation and clinical significance of LGI1 in ESCC. (A) Downregulation of LGI1 was frequently detected in primary ESCCs by qRT–PCR (left) and western blot analysis (right). N, non-tumor tissue; T, tumor tissue; ***P < 0.001, paired Student’s t-test. (B) Absent or downregulation of LGI1 was detected in nine ESCC cell lines by qRT–PCR (left) and western blot analysis (right). A pooled normal tissue control was used as control. (C) Representative of LGI1 expression in a pair of adjacent non-tumor (upper) and tumor tissue (lower) detected by immunostaining with anti-LGI1 antibody (brown). The slide was counterstained with hematoxylin (original magnification ×200). (D) Kaplan–Meier curves for DSS of patients with ESCC according to the expression status of LGI1. Blue, patients with normal expression of LGI1 (n = 111, median survival 31 months); Green, patients with low expression of LGI1 (n = 113, median survival 18 months, P < 0.001, log-rank test). (E) Stratified survival analysis according to the pathological stage. LGI1 expression could differentiate the prognosis of patients both in early clinical stage (pStage I and II; P = 0.001, log-rank test; left) and in advanced clinical stage (pStage III; P < 0.001, log-rank test; right).
ESCC tissue microarray and immunohistochemical staining

A total of 300 formalin-fixed, paraffin-embedded ESCC tumor specimens and the corresponding normal epithelia were selected from Linzhou Cancer Hospital (Henan, China). The ESCC tissue microarray (TMA) was constructed as described previously (17). Briefly, tissue sections with 5 μm thick were cut from the TMA blocks and mounted on microscope slides. For immunohistochemical (IHC) analysis, the slides were deparaffinized, rehydrated, blocked by 10% normal goat serum at room temperature for 30 min. The slides were then incubated with rabbit polyclonal antibody against LGI1 (Sigma–Aldrich) at a dilution of 1:100 at 4°C overnight and subsequently incubated with biotinylated goat anti-rabbit immunoglobulin at a concentration of 1:50 for 30 min at 37°C. A staining index (values 0–7) was calculated by adding the scores for the intensity of LGI1-positive staining (negative, 0; weak, 1; moderate, 2; strong, 3) and the percentage of LGI1-positive cells (<25%, 1; 25–50%, 2; >50–75%, 3; >75%, 4).

Plasmid transfection

The human LGI1 cDNA was PCR amplified, cloned into pcDNA3.1(+) and transfected into ESCC cell line cells KYSE30 and KYSE510 using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instruction. Stable LGI1-transfected clones (LGI1-30/LGI1-510) were selected with 500 μg/ml of G418. Empty vector-transfected KYSE30 and KYSE510 cells (Vec-30/Vek-510) were used as controls.

Lentiviral transduction

LGI1 small hairpin RNA (shRNA) lentiviral knockdowns (GeneCopoeia NM_005097.1) or shRNA non-target control (NTC) were packaged using HIV-based packaging mix (GeneCopoeia) and used to infect LGI1-30 and LGI1-510 cells to establish cells constitutively repressing LGI1. Stable clones were selected using puromycin. Cells were infected with lentiviral media at a multiplicity of infection of 10, in the presence of 5μg/ml polybrene (Sigma–Aldrich) overnight in a 37°C incubator.

Cell growth and foci formation assays

To perform growth assay, cells were seeded at a density of 1000 cells per well in 96-well plate. The cell growth rate was detected using Cell Counting Kit-8 (CCK8; Dojindo Molecular Technologies) according to the manufacturer’s instruction. Triplicate independent experiments were carried out. For foci formation assay, 1000 cells were seeded in each well of a six-well plate. After 12 days of culture, surviving colonies were counted with crystal violet staining. Triplicate independent experiments were carried out.

Tumor formation assay in nude mice

LGI1- and Vec-transfected cells (2x10⁶) were injected into the left and right dorsal flank of 5-week-old athymic nude mice, respectively. Tumor formation in nude mice was monitored over a 4 week period. The tumor volume was calculated by the formula V = 0.5 x L x W².

Transwell cell migration and invasion assays

The transwell cell migration assay and invasion assay were performed using BD chambers containing polyethylene terephthalate membranes of 8 μm pore size and BD Matrigel Invasion Chambers with 8 μm porosity (BD Biosciences, Bedford, MA) according to the manufacturer’s instructions, respectively. Cells were suspended in serum-free RPMI 1640 at a density of 8.0 x 10⁴ cells per well onto the upper chambers and RPMI 1640 with 10% fetal bovine serum was added to the lower chambers. Cells that had migrated through to the bottom of the insert membrane were fixed, stained and counted from six random fields under a x20 objective lens. The experiments were repeated three times.

Western blot analysis

Western blotting was done according to the standard protocol with antibodies for rabbit anti-LGI1 (Sigma–Aldrich; 1:1000), anti-MMP-3 (Epitomic; 1:500) and β-tubulin (Cell Signaling Technology; 1:1000); and Phospho-Erk1/2 Pathway Sampler Kit, Phospho-p38 MAPK Pathway Sampler Kit and Phospho-Akt Pathway Sampler Kit (Cell Signaling Technology; 1:1000).

Statistical analysis

Statistical analyses were performed using the SPSS 16.0. Results were expressed as mean ± standard error of the mean. The statistical comparisons between groups were analyzed using the Student’s t-test. Differences in LGI1 expression among different clinicalopathological stages were analyzed by paired sample t-test and Chi-squared test (χ² test). The disease-specific survival (DSS) time, which was defined as the time from surgery to death (living patients were censored at the time of their last follow-ups) of ESCC patients with different LGI1 expression levels was estimated by Kaplan–Meier analysis and log-rank test. Univariate and multivariate survival analyses were performed using the Cox proportional hazard model with a forward stepwise procedure (the entry and removal probabilities were 0.05 and 0.10, respectively). A significant difference was considered statistically when P value was <0.05.

Results

LGI1 is frequently downregulated in ESCC

The messenger RNA (mRNA) expression of LGI1 was initially tested in 50 pairs of primary ESCC tumors and their corresponding non-tumorous tissues by qRT–PCR. The relative expression level of LGI1 was significantly downregulated in tumor tissues compared with their non-tumor counterparts (P < 0.001, Figure 1A). Downregulation of LGI1 was detected in 24/50 (48.0%) of ESCC tumors compared with their normal counterparts (defined as a 2-fold decrease of LGI1 expression in tumors). Western blotting showed that downregulation of LGI1 protein was detected in 21/40 (52.5%) of randomly selected ESCCs (Figure 1A). LGI1 expression was also examined in nine ESCC cell lines by qRT–PCR and western blotting. As compared with a pooled normal tissue control, downregulation of LGI1 was detected in all nine ESCC cell lines (Figure 1B).

LGI1 downregulation is associated with ESCC metastasis and poor prognosis

LGI1 expression was further studied in a large scale of ESCCs by IHC using a TMA containing 300 pairs of primary ESCCs. Informative IHC results were obtained from 224 pairs of ESCCs. Non-informative samples included lost samples, unrepresentative samples and samples with too few tumor cells (<100 cells per case), which were excluded in data compilation. Because the staining index of LGI1 in non-tumor tissue was equal or larger than 5, staining index ≥5 was then counted as normal expression of LGI1. Downregulation of LGI1 was defined when staining index was <5. Downregulation of LGI1 was detected in 113/224 (50.5%) of informative ESCC tissues compared with their adjacent non-tumor tissues (Figure 1C).

The correlation between LGI1 expression status and clinicopathologic features of ESCC was further evaluated. The results showed that downregulation of LGI1 was significantly associated with advanced pathologic T category (P = 0.009), pathologic N category (P < 0.001) and clinical stage (P < 0.001, Table I). No correlation was observed between LGI1 expression and patient’s age (P = 0.679), gender (P = 0.589) and tumor cell histological differentiation (P = 0.454).

Table I. Association of LGI1 expression with clinicopathological features in ESCCs

<table>
<thead>
<tr>
<th>Clinical features</th>
<th>Cases</th>
<th>LGI1 expression</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Low level (%)</td>
<td>Normal level (%)</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤59</td>
<td>122</td>
<td>60 (49.2%)</td>
<td>62 (50.8%)</td>
</tr>
<tr>
<td>&gt;59</td>
<td>102</td>
<td>53 (52.0%)</td>
<td>49 (48.0%)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>128</td>
<td>67 (52.3%)</td>
<td>61 (47.7%)</td>
</tr>
<tr>
<td>Female</td>
<td>96</td>
<td>46 (47.9%)</td>
<td>50 (52.1%)</td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Grade 1</td>
<td>24</td>
<td>10 (41.7%)</td>
<td>14 (58.3%)</td>
</tr>
<tr>
<td>Grade 2</td>
<td>147</td>
<td>73 (49.7%)</td>
<td>74 (50.3%)</td>
</tr>
<tr>
<td>Grade 3</td>
<td>53</td>
<td>30 (56.6%)</td>
<td>23 (43.4%)</td>
</tr>
<tr>
<td>pT category</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>26</td>
<td>9 (34.6%)</td>
<td>17 (65.4%)</td>
</tr>
<tr>
<td>T2</td>
<td>49</td>
<td>18 (36.7%)</td>
<td>31 (63.3%)</td>
</tr>
<tr>
<td>T4</td>
<td>149</td>
<td>86 (57.7%)</td>
<td>63 (42.3%)</td>
</tr>
<tr>
<td>pN category</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>N0</td>
<td>125</td>
<td>45 (36.0%)</td>
<td>80 (64.0%)</td>
</tr>
<tr>
<td>N1-3</td>
<td>99</td>
<td>68 (68.7%)</td>
<td>31 (31.3%)</td>
</tr>
<tr>
<td>Clinical stage</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Early (I and II)</td>
<td>150</td>
<td>56 (37.3%)</td>
<td>94 (62.7%)</td>
</tr>
<tr>
<td>Advanced (III)</td>
<td>74</td>
<td>77 (70.7%)</td>
<td>23 (29.3%)</td>
</tr>
</tbody>
</table>

Statistical significance (P < 0.05) is shown in bold.
Kaplan–Meier analysis showed that the 3 year DSS rate was significantly lower in ESCC patients with LGI1 downregulation (12.4%) than that with normal LGI1 expression (48.6%, \(P < 0.001\), log-rank test, Figure 1D). In a stratified survival analysis according to the pathological stage, downregulation of LGI1 could effectively predict poorer prognosis of ESCCs both in early clinical stage (pStage I and II; \(P = 0.001\), log-rank test, Figure 1E) and advanced clinical stage (pStage III; \(P < 0.001\), log-rank test, Figure 1E). Further, by multivariate survival analysis including pathologic T category, pathologic N category and LGI1 expression, which had impact on survival of patients, we found that pathologic N category (\(P = 0.021\)) and expression of LGI1 (\(P < 0.001\)) were two independent prognostic predictors for resected ESCC patients enrolled in this study (Supplementary Table S2, available at Carcinogenesis Online).

To assess the tumor suppressive ability of LGI1, LGI1 was stably transfected into KYSE30 and KYSE510 cells (LGI1-30 and LGI1-510). Empty vector-transfected cells were used as controls (Vec-30 and Vec-510). Expression of LGI1 was confirmed at both mRNA and protein levels by qRT–PCR and western blot analysis (Figure 2A). In both in vitro and in vivo functional assays were applied to characterize the tumor suppressive ability of LGI1. Compared with control cells, LGI1-transfected cells exhibited reduced growth rates (\(P < 0.01\), Figure 2B) and lower foci formation frequencies (\(P < 0.001\), Figure 2C). Tumor formation in nude mice was further used to test the in vivo tumor suppressive role of LGI1. The result found that tumor formation ability of LGI1-transfected cells was significantly subdued as compared...
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Furthermore, we found that knockdown of LGI1 by shRNAs against LGI1 (sh1 or sh2) in LGI1-30 and LGI1-510 cells (Figure 3A) could significantly promote cell growth rate (P < 0.01, Figure 3B) and foci formation efficiency, compared with NTC cells (P < 0.001, Figure 3C). Collectively, these data indicate that LGI1 possesses a strong tumor suppressive role both in vitro and in vivo.

LGI1 suppresses cell motility and invasiveness

As the TMA result indicated that downregulation of LGI1 was significantly associated with ESCC metastasis, the effects of LGI1 on cell migratory and invasive capabilities were further investigated using cell migration and invasion assays. Both cell migration and invasion assays found that LGI1-transfected cells led to a significant reduction in the abilities of cell migration and invasion (P < 0.001, Figure 4A). Conversely, knockdown of LGI1 in LGI1-30 and LGI1-510 cells yielded opposing effects (P < 0.001, Figure 4B).

LGI1 downregulates MMP-3 via extracellular signal-regulated kinase signaling pathways

Numerous studies have demonstrated that elevated expression of MMPs is responsible for tumor invasion and metastasis (18). To explore whether the effect of LGI1 on cell motility is associated with downregulation of MMPs, expression levels of MMPs (MMP-1, -2, -3, -7, -9, -10 and -13) were compared between LGI1- and empty vector-transfected cells by qRT–PCR. The results showed that only expression of MMP-3 was significantly decreased in LGI1-transfected cells compared with control cells (P < 0.01, Figure 5A). However, no obvious changes were detected in MMP-1, -2, -3, -7, -9, -10 and -13. In addition, knockdown of LGI1 in LGI1-30 and LGI1-510 cells rescued the expression of MMP-3 (Figure 5B). To assess the correlation between LGI1 and MMP-3 expression, as well as clinical significance of MMP-3 in ESCC, expression of MMP-3 was examined by IHC using the same ESCC TMA. Informative results were obtained from 236 cases including all 224 informative cases detected by LGI1.
Correlation study showed that downregulation of LGI1 was significantly associated with elevated expression of MMP-3 \((P < 0.001, \text{Supplementary Table S3, available at Carcinogenesis Online})\). As shown in Supplementary Table S4, available at Carcinogenesis Online, high expression of MMP-3 was significantly associated with advanced pathologic N category \((P = 0.016)\) and clinical stage \((P = 0.002)\). Moreover, Kaplan–Meier survival curve showed that high expression of MMP-3 was significantly correlated with shorter DSS of patients \((P = 0.020, \text{log-rank test, Supplementary Figure S1, available at Carcinogenesis Online})\).

Mitogen-activated protein kinase (MAPK) signaling pathway, including well-known mediators extracellular signal-regulated kinase (ERK1/2) and p38MAPK, can stimulate or inhibit MMPs expression depending on cell types \((19,20)\). To further investigate the molecular mechanism by which LGI1 regulates MMP-3 expression, we studied the effects of LGI1 on the activation of several potential signaling pathways including ERK1/2 and p38MAPK pathways. Our data showed that the expression level of phosphorylated ERK1/2 was dramatically decreased in LGI1-transfected cells and increased in LGI1-silenced cells, whereas the expression level of total ERK was unaffected.

**Fig. 4.** LGII inhibits cell motility and invasiveness. (A) Transwell cell migration and invasion assays were used to compare cell migration and invasion between LGII- and vector-transfected cells. The cells that migrated through the polyethylene terephthalate membrane or invaded through the Matrigel were fixed and stained with crystal violet (original magnification x200). The results were expressed as mean ± SD of three independent experiments. ***\(P < 0.001\), Student’s \(t\)-test. (B) Transwell cell migration and invasion assays were used to compare cell migration and invasion between LGII-transfected cells treated with NTC shRNA or shRNA against LGII (sh1 and sh2). The cells that migrated through the polyethylene terephthalate membrane or invaded through the matrigel were fixed and stained with crystal violet (original magnification x200). The results are expressed as mean ± SD of three independent experiments. ***\(P < 0.001\), Student’s \(t\)-test.
Figure 5C. For p38MAPK, no obvious changes were detected in both total and phosphorylated proteins (Figure 5C). These data suggest that LGI1 may regulate the expression of MMP-3 transcription via the ERK1/2 pathway. In some circumstances, PI3K/Akt signaling pathway has been shown to be involved in the control of Raf-MEK-ERK pathway (21). However, in this study, the expression of total and phosphorylated Akt remained unchanged in LGI1-transfected or -silenced cells.

To further confirm the importance of ERK1/2 in LGI1-modulating MMP-3 expression, MEK1/2 inhibitor U0126 was applied to inhibit the activity of ERK1/2 in LGI1- and Vec-transfected cells. Treatment with U0126 severely diminished phosphorylation level of ERK1/2 in the LGI1- and Vec-transfected cells, leading to the downregulation of MMP-3 (Figure 5D). This result confirmed that the downregulation effect of LGI1 on MMP-3 was through the ERK1/2 pathway. It has been reported that the protein kinase C activator phorbol myristate acetate (PMA) can activate the MEK–ERK signaling pathway (22). We next tested whether protein kinase C was involved in the LGI1–ERK1/2–MMP-3 signaling by treating LGI1- and Vec-transfected cells with PMA. Treatment with PMA stimulated significant increase in ERK1/2 phosphorylation level accompanied with MMP-3 production in LGI1-transfected cells, whereas modest one in control cells, suggesting that the decline of MMP-3 production mediated by LGI1 could be reversed by activation of protein kinase C (Figure 5D).

Discussion

The LGI1 gene, located in 10q24, was originally isolated at a breakpoint of a reciprocal chromosomal translocation t(10;19) in a glioblastoma cell line T98G and has been reported as a tumor suppressor gene in brain tumor (11,23,24). However, the biological function of LGI1 is largely unrevealed in ESCC. In this study, we first demonstrated that LGI1 was frequently downregulated in ESCC, which was associated with tumor metastasis and poor outcome of ESCC patients. Both in vitro and in vivo assays demonstrated that LGI1 had strong tumor suppressive function. The results showed that LGI1 could effectively suppress cell growth rate, foci formation and tumor formation in nude mice. In addition to its tumor suppressive roles, this study also demonstrated that LGI1 could inhibit ESCC cell motility and invasiveness. The MMPs, best characterized for degrading the extracellular matrix and basement membrane, play a critical role in tumor invasion and metastasis. Overexpression of MMP-2, 3, 7, 9 and 10 in tumor tissues of ESCC patients has been shown in several studies (25–27). Here, we analyzed the regulatory effects of LGI1 on several MMPs and found that only MMP-3 could be downregulated by LGI1. The correlation analysis in 224 ESCC tissues confirmed that downregulation of LGI1 was significantly associated with high expression of MMP-3. Besides, the genetic-clinopathologic correlation analysis indicated that downregulation of LGI1 was observed more frequently in patients with tumor invasion and lymph node metastasis. These results are interesting in light of the fact that MMP-3 plays a crucial role in degradation of extracellular matrix, as well as lymphatic invasion and lymph node metastases (28–30). Further, molecular studies suggest that the suppression effect of LGI1 on MMP-3 production in vitro is exerted through downregulation of the ERK1/2 pathway, in line with the previous study on glioblastoma cells (24). MAPKs have been reported to be able to affect MMP-3 production directly by influencing the activation of the AP-1 element that regulates the expression of MMP-3 (19). In light with these findings, we suggest that LGI1 is a tumor modulator in ESCC metastasis through deregulated ERK1/2 pathway. On the other hand, the previous study on glioblastoma implied that LGI1 might impair the activation of the ERK1/2 pathway through Akt phosphorylation, resulting in downregulation of MMP-3 (24). However, our results did not show the influence of LGI1 on the Akt signaling pathways in ESCC.
Furthermore, the PMA-induced reversal of LGI1-mediated suppression of ERK1/2 phosphorylation, as well as MMP-3 production, implicates that LGI1 may be involved in the procedure of transducing extracellular signals. The LGI1 gene contains a number of specific motifs that give clues to its potential function. At the N-terminus, a signal peptide involving the first 35 amino acids and a putative transmembrane domain imply that this protein is transferred to the membrane and contains intracellular and extracellular components. In the extracellular domain, 3.5 tandemly arrayed LRRs flanked on both sides by conserved cysteine clusters (11). The C-terminal half of the protein consists of seven epi-
lepdy-associated repeats, or epitempin repeats (31,32). Both LRR and epipell-episituated repeats/epitope domains are involved in protein–
protein interaction or specific receptor–ligand binding. In light of this investigation, we postulate that LGI1 may transfer to the membrane and mediate signals from cell membrane receptors triggered by growth factors, cytokines, hormones, and cell–cell and cell–matrix interactions. Of interesting, recent findings suggest that LGI1 is a secreted protein and reduces epidermal growth factor receptor protein levels resulting in downregulation of the ERK1/2 pathway and an inhibition of cell motility (33–35). Further studies will be necessary to elucidate the tumor suppressive mechanism of LGI1 on ERK1/2 signaling pathways.

Mechanisms of silencing of tumor suppressor genes include the following: mutation, loss of heterozygosity, alteration in CpG island methylation patterns and histone modifications. Our preliminary study suggests that the downregulation of LGI1 does not seem to associate with loss of heterozygosity, methylation of the LGI1 promoter or histone modifications (Supplementary Figure S2, available at Carcinogenesis Online). Nevertheless, further investigation is still needed to confirm these findings. Although a previous study did not identify any muta-
tions within the coding or promoter region of LGI1 in glioma-derived cells, we cannot formally rule out the possibility that mutations lead to decreased mRNA of LGI1 in ESCC (36). Future studies should attempt to unravel the mechanisms of LGI1 inactivation in ESCC tumorigenesis.

In summary, our findings demonstrate that LGI1 is a novel tumor suppressor gene and plays an important role in the pathogenesis and metastasis of ESCC. A better understanding of the tumor suppressive mechanism of LGI1 during ESCC initiation and progression may pro-
vide a novel therapeutic strategy to ESCC patients.

Supplementary material
Supplementary Table S1–S4 and Figures S1 and S2 can be found at http://carcin.oxfordjournals.org/

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