Overexpression of LRIG1 regulates PTEN via MAPK/MEK signaling pathway in esophageal squamous cell carcinoma

XIAOFANG JIANG¹ and HUIWU LI²,³

¹Central Laboratory; ²Department of Biochemistry, School of Basic Medicine; ³Tumor Institute, Affiliated Tumor Hospital, Xinjiang Medical University, Urumqi, Xinjiang 830011, P.R. China

Received April 8, 2016; Accepted June 20, 2016

DOI: 10.3892/etm.2016.3606

Abstract. The present study aimed to evaluate the role of leucine-rich repeats and immunoglobulin-like domain protein 1 (LRIG1) in the regulation of phosphatase and tensin homolog (PTEN) expression in esophageal carcinogenesis. LRIG1 was overexpressed in esophageal squamous cell carcinoma (ESCC) cell lines, and the effect of LRIG1 overexpression on the mRNA and protein expression levels of PTEN was evaluated by reverse transcription-quantitative polymerase chain reaction and western blotting. Furthermore, the effects of LRIG1 overexpression on the cell cycle distribution and apoptosis of ESCC cells were examined by flow cytometry. Various cell signaling pathway inhibitors were used to assess the effects of LRIG1 on downstream signaling in ESCC cell lines. In addition, the association between LRIG1 and PTEN expression was examined in 48 samples from patients with ESCC. LRIG1 overexpression was demonstrated to downregulate PTEN expression in ESCC cell lines, and promote their proliferation and inhibit apoptosis. In addition, LRIG1-mediated suppression of PTEN expression was inhibited by the U0126 inhibitor, which suggests that LRIG1 may inhibit the activation of PTEN signaling molecules by triggering the mitogen-activated protein kinase (MAPK)/MAPK kinase 1 (MEK) signaling pathway. In conclusion, the present study demonstrated that overexpression of LRIG1 significantly and adversely affected the survival of ESCC cells, and that the MAPK/MEK signaling pathway may be responsible for the repression of PTEN expression and function.

Introduction
Leucine-rich repeats and immunoglobulin-like domain protein 1 (LRIG1) is located at chromosome 3p14, which is recurrently deleted in human cancers (1,2). A previous study demonstrated that LRIG1 was a negative regulating factor of numerous oncogenic receptor tyrosine kinases (RTKs) (3). In two independent studies, LRIG1 was shown to downregulate the expression of four members of the epidermal growth factor (EGF) family of receptor tyrosine kinases (ERBB receptors), including the EGF receptor (EGFR), human epidermal growth factor receptor 2 (HER2), HER3 and HER4 (3,4). In addition, LRIG1 was shown to reduce the levels of EGFR by accelerating receptor ubiquitylation and lysosomal degradation (4). Furthermore, LRIG1 has been reported to regulate ERBB receptor degradation and act as a tumor suppressor (5). However, at present, no correlation has been demonstrated between LRIG1 and EGFR expression in colorectal cancer (6). Thus, the various functions of LRIG1 in cancer remain uncertain.

Previous studies reported that LRIG1 overexpression suppressed the growth of the PC3 prostate cancer cell line, and that this effect was overcome by androgen in patients with increased LRIG1 and androgen activities (7,8). These findings suggested that LRIG1 may be unable to elicit sufficient tumor suppressive activity in a high-androgen environment. The prognostic significance of the LRIG1 protein in cervical cancer has been associated with tumor suppressors, oncogenes and numerous factors, including cancer subtype and stage (9). LRIG1 expression has been shown to be downregulated in a number of cancer types, although it was reported to be overexpressed in prostate and colorectal tumors (7,8). The expression level and subcellular location of LRIG proteins have a prognostic value in brain tumors (10). Due to this heterogeneity in results, whether the function of LRIG1 suppresses or promotes tumor growth remains unclear.

Phosphatase and tensin homolog (PTEN) is a type I protein tyrosine phosphatase containing five domains. The phosphatase and tensin domains of PTEN are recurrently deleted from chromosome 10q23 in oncogenic events (11). PTEN targets a wide range of molecules, thereby regulating tumorigenic functions such as apoptosis, the cell cycle, cell adhesion and cell migration (12). The function of LRIG1 and
its regulatory association with the PTEN gene in esophageal squamous cell carcinoma (ESCC) remains unclear. Therefore, the present study aimed to investigate the association between LRIG1 and PTEN in ESCC cell lines and patient samples.

Materials and methods

Reagents. The pEGFP-N1-LRIG1 plasmid was provided by Professor Guo Dongsheng (Huazhong University of Science & Technology, Wuhan, China). The empty pEGFP-N1 vector, used as a control, was provided by Xinjiang Medical University (Urumqi, China). Inhibitors of phosphoinositide 3-kinase (PI3K; LY294002), mitogen-activated protein kinase (MAPK) kinase 1 (MEK; PD98059, U0126), p38-MAPK2 (SB203580), janus kinase 2 (JAK2; AG-490), Ca2+/calmodulin-dependent protein kinase II (KN-62) and calcineurin (FK-506) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The protein kinase C (PKC) inhibitor, bisindolylmaleimide I, was obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-LRIG1 antibody was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Anti-PTEN antibody was purchased from Wuhan Boster Biological Technology, Ltd. (Wuhan, China).

Cell culture. Eca-109 human esophageal cancer cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). KYSE-450 human esophageal cancer cells were purchased from the Beijing Institute for Cancer Research (Beijing Cancer Hospital, Beijing, China). All cells were cultured at 37˚C in an 5% CO2 humidified incubator. The empty pEGFP-N1, according to the manufacturer's protocols. Briefly, the cells were cultured at a density of 4x104 in Roswell Park Memorial Institute-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 5% fetal bovine serum (Hanzhou Siijiqing Biological Engineering Materials Co., Ltd., Hangzhou, China) and 100 µg/ml penicillin/streptomycin in six-well plates in a 5% CO2 humidified incubator at 37˚C. After achieving 80% confluence, the Eca-109 and KYSE-450 cells were pretreated with LY294002 (10 µM), PD98059 (50 µM), U0126 (10 µM), bisindolylmaleimide I (4 µM), SB203580 (10 µM), AG-490 (10 µM), KN-62 (10 µM) and FK-506 (10 µM) for 1 h at 37˚C, followed by transfection with pEGFP-N1-LRIG1 or pEGFP-N1 (control) plasmids using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 48 h.

Patients. A total of 48 pairs of ESCC and nearby normal esophageal tissue samples were obtained from Hazak patients at the Affiliated Tumor Hospital of Xinjiang Medical University between January 2008 and June 2009. Informed consent was obtained from all patients, and the present study was approved by the research ethical committee of the Affiliated Tumor Hospital of Xinjiang Medical University. All patients underwent a radical esophagectomy without preoperative chemotherapy or radiotherapy, and the pathological diagnosis of ESCC was confirmed by histopathological analyses. Tumors were classified as stage I (n=1), IIa (n=13), IIb (n=5) or III (n=29), according to the criteria of the Union Internationale Contra Cancrum tumor-node-metastasis classification system (13) of malignant tumors. Tumors and coupled normal esophageal tissues from the same patient were harvested and analyzed by senior pathologists, followed by freezing in liquid nitrogen within 30 min postoperatively and storing at -80˚C until experimental use.

Immunohistochemical analysis. Cells (4x104) were cultured in RPMI-1640 supplemented with 5% fetal bovine serum and 100 µ/ml penicillin/streptomycin in six-well plates. The plates were then placed in a 5% CO2 humidified incubator at 37˚C. Following 24 h of growth, cells attached to the glass slide were fixed with 4% poly formaldehyde and counted by immunohistochemical staining. Further steps were conducted in a hydrated chamber at room temperature. Endogenous peroxidase activity was inhibited using a peroxidase block (Wuhan Boster Biological Technology, Ltd.) for 5 min, after which the slides were incubated with 20% normal goat serum in 50 mM Tris-HCl (pH 7.4). The cells attached to the glass slide were incubated with goat anti-human LRIG1 monoclonal antibody (1:100) overnight at 4˚C. Subsequently, the cells were incubated with rabbit horseradish peroxidase (HRP)-conjugated anti-goat IgG secondary antibody (1:6,000; cat. no. BA1060; Wuhan Boster Biological Technology, Ltd.) at 37˚C for 30 min, following washing with phosphate-buffered saline (PBS), and stained with 3,3'-diaminobenzidine. The slides were visualized by fluorescent inverted microscopy.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the tissue samples and ESCC cell lines using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. cDNA was synthesized from 1 µg RNA in a 20 µl reaction mixture using the Reverse Transcription System (cat. no. A3500; Promega Corporation, Madison, WI, USA). cDNA was amplified using the following primers: GAPDH forward, 5'-GACCTGACCTGCCGCATA-3' and reverse, 5'-AGGAATTGTGGTCTGCTG-3'; LRIG1 forward, 5'-ACCTGTGACACCTGGAGAAT-3' and reverse, 5'-TCATCGCAGAATGAGTCCTC-3'; and PTEN forward, 5'-AAAGGAGCAGAATGTGGTGTGTAATG-3' and reverse, 5'-TGCTCCTTACTTCCCACAGAA-3'. qPCR was performed using the SYBR Green PCR Master Mix (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. PCR was performed in a reaction volume of 20 µl containing 10 µl 2X PCR Master, 2 µl cDNA, 7 µl water, 0.5 µl forward primer and 0.5 µl reverse primer. The thermal cycling conditions were as follows: 5 min at 95˚C, followed by 40 cycles (94˚C, 30 s; 55˚C, 30 s; 72˚C, 30 s). Relative mRNA expression levels were determined by normalization to GAPDH using the 2-ΔΔCT method (14).

Western blot analysis. Cells and tissues (following tissue homogenization) were lysed using cell lysis buffer (5 mM Tris-HCl, 1 mM EGTA, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.1% SDS and 1 mM PMSF; pH 7.5) containing 1% Triton X-100 with protease inhibitors at 4˚C for 30 min. Protein lysates were purified by centrifugation (15,000 x g; 4˚C, 10 min), after which equal quantities of protein (60 µg) were separated from various cells and tissue samples and boiled for 5 min. The proteins were then separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes.
Results

**LRIG1 downregulates PTEN expression in esophageal cancer cell lines.** The pEGFP-N1-LRIG1 plasmid was transfected into Eca-109 and KYSE-450 cell lines, in which LRIG1 expression levels were intrinsically low. LRIG1 expression was inversely correlated with PTEN expression in Eca-109 and KYSE-450 cell lines following transfection of the cells. At 48 h after transfection, fluorescence microscopy detected green fluorescence in the transfected cells (Fig. 1A and B). The mRNA expression levels of LRIG1 were examined by RT-qPCR at 48 h following transfection, and demonstrated a significant increase (magnitude, >215) in the expression levels of LRIG1 mRNA in the transfected cells, as compared with the negative controls (Fig. 1C). In addition, western blotting demonstrated that the protein expression levels of LRIG1 were significantly increased by ~10.1% in KYSE-450 cells and 50.5% in Eca-109 cells transfected with pEGFP-N1-LRIG1 plasmid, as compared with the negative controls (P<0.05; Fig. 1D).

RT-qPCR and western blotting demonstrated a correlation between PTEN and LRIG1 expression levels in Eca-109 and KYSE-450 cell lines following transfection with the pLRIG1-GFP-N1 plasmid (Fig. 1C and D). Upregulation of LRIG1 expression markedly decreased the mRNA and significantly decreased the protein expression levels (P<0.05) of PTEN in the transfected cells (Fig. 1C and D), as compared with the negative controls.

The cells were pretreated with various inhibitors to elucidate the signal transduction pathways involved in the LRIG1-mediated inhibition of PTEN expression. The ability of LRIG1 to downregulate PTEN expression was diminished by pretreatment of Eca-109 and KYSE-450 cells with U0126 and KN-62 inhibitors (Fig. 2). These results suggest that LRIG1-induced downregulation of PTEN in esophageal cancer cells involves the MEK signaling pathway.

**Overexpression of LRIG1 reduces apoptosis in Eca-109 and KYSE-450 cells.** The effect of LRIG1 overexpression on the apoptosis of Eca-109 and KYSE-450 cells transfected with the pEGFP-N1-LRIG1 plasmid was assessed by flow cytometry. The percentage of apoptotic Eca-109 cells was significantly decreased from 41.70% in control cells to 19.47% in overexpressing cells (P<0.05; Fig. 3). Similarly, the percentage of apoptosis of Eca-109 and KYSE-450 cells transfected with pEGFP-N1-LRIG1 plasmid, as compared with the negative controls (P<0.05; Fig. 3).

Overexpression of LRIG1 arrested the cell cycle in the S-phase. The effects of LRIG1 overexpression on the cell cycle distribution of Eca-109 and KYSE-450 cells was assessed by flow cytometry (Fig. 4A-C). As compared with the control cells, LRIG1 overexpression increased the percentage of Eca-109 and KYSE-450 cells in the S-phase of the cell cycle. The percentage of Eca-109 cells in the S-phase was increased from 42.11% of the control cells to 47.19% of the LRIG1 overexpressing cells (P<0.05; Fig. 4D). Similarly, the percentage of KYSE-450 cells in the S-phase was increased from 58.64% of the control cells to 71.24% of the LRIG1 overexpression cells (Fig. 4D).
LRIG1 protein localization in Eca-109 and KYSE-450 cells. The localization of LRIG1 in Eca-109 and KYSE-450 cells was determined by immunohistochemical analyses. The perinuclear staining of LRIG1 was observed by microscopy in Eca-109 cells following transfection of the cells with the pLRIG1-GFP plasmid. In addition, the LRIG1 protein was primarily observed in the cytoplasm of KYSE-450 cells (Fig. 5).

PTEN mRNA expression negatively correlated with LRIG1 in cancerous tissue. Total RNA was isolated from ESCC tissues and corresponding normal distant tissues from the
same patient, and the mRNA expression levels of \textit{LRIG1} and \textit{PTEN} were determined by RT-qPCR. The mRNA expression levels of \textit{PTEN} were inversely correlated to the expression levels of \textit{LRIG1} in the cancerous tissue ($R=0.478$; $P=0.002$; Table I). No significant correlation was observed between clinical pathological factors and the expression levels of \textit{LRIG1} or \textit{PTEN}.

### Discussion

\textit{LRIG1}, a membrane-associated protein, has been shown to inhibit growth factor signal transduction from oncogenic RTKs, including EGFR, and MET and RET proto-oncogenes (15-17). The downregulation of \textit{LRIG1} expression results in spontaneous tumor formation, which suggests that
LRIG1 may serve as a tumor suppressor in certain types of cancer (2). However, LRIG1 has been shown to be overexpressed in numerous cancers, including leukemia, astrocytoma and prostate cancers, thus suggesting that LRIG1 may perform general downregulation in all types of human tumors (7,18), and that the role of LRIG1 may be masked by other factors. Therefore, the expression and function of LRIG1 should be carefully evaluated. In a previous study performed by the authors, it was demonstrated that LRIG1 expression was not associated with EGFR expression, although it was correlated with HER2 expression in ESCC (19), which was consistent with the demonstrated correlation between increased gene copy numbers of HER2 and LRIG1 in a previous study (20).

The progression of esophageal cancer has been associated with the loss of tumor suppressor genes, including PTEN (21). PTEN may be an important biological marker in the treatment of human esophageal cancer (21). The present study demonstrated a significant correlation between LRIG1 and PTEN mRNA expression levels in ESCC cell lines, and in tumor samples from patients with ESCC. Notably, LRIG1 overexpression downregulated PTEN expression in Eca-109 and KYSE-450 cell lines, indicating that LRIG1 may have an oncogenic role. The expression and subcellular localization of LRIG1 may be associated with specific clinicopathological features of ependymoma tumors, and thus may be of great importance in carcinogenesis (22). In human oligodendroglioma, the cytoplasmic and perinuclear localizations of LRIG1 were associated with the expression of various genes; thus suggesting that LRIG1 may perform various functions. In the present study, immunohistochemical analysis demonstrated that LRIG1 was predominantly expressed in the cytoplasm in ESCC cell lines. Notably, a number of perinuclear LRIG1 were observed following transfection with LRIG1 plasmid.

PTEN selectively inhibits the activation of extracellular signal-regulated kinase (ERK) in the MAPK signaling pathway (23,24). The absence of PTEN in cancer cells has typically been associated with the increased activation of the PI3K/Akt signaling pathway, leading to malignant cancer transformation and progression (25,26). In the present study, LRIG1-mediated downregulation of PTEN mRNA expression was diminished by pretreatment of ESCC cell lines with bisindolylmaleimide I (PKC inhibitor), PD98059 (ERK 1/2 inhibitor) and U0126 (MEK 1/2 inhibitor). These results suggest that LRIG1 regulates PTEN expression via the MAPK/MEK/ERK signaling pathway.

The apoptosis of Eca-109 and KYSE-450 cells was significantly inhibited following transfection of the cells with the pLRIG1-EGF plasmid, compared with the control cells. Furthermore, LRIG1 overexpression increased the percentage of Eca-109 and KYSE-450 cells in the S-phase of the cell cycle. These results suggested that LRIG1 inhibited cell apoptosis and promoted the growth of cancer cells, serving as an oncogene in ESCC. In addition, these results
indicated that LRIG1 may function as an oncogene by regulating tumor-suppressor genes in a manner that is dependent on the subcellular localization of LRIG1. The overexpression of cytoplasmic LRIG1 in ESCC cell lines may have promoted cell growth by suppressing apoptosis and accelerating the cell cycle.

In conclusion, the present study demonstrated that LRIG1 overexpression downregulated PTEN expression in ESCC cell lines by activating the MAPK/MEK signaling pathway. In addition, LRIG1 was shown to function as a proto-oncogene by promoting tumor cell proliferation and simultaneously decreasing cellular apoptosis. LRIG1
followed a non-canonical mechanism of interaction with various signal transduction pathways to elicit novel functions in the cytoplasm of tumor cells.

Acknowledgements

The present study was supported by the Nature Science Foundation of China (grant nos. 30950012 and 81360304) and the Xinjiang Endemic Molecular Biology Laboratory, China (grant no. xjd2002-2011-02).

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