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LRIG1, a 3p tumor suppressor, represses EGFR signaling and is a novel epigenetic silenced gene in colorectal cancer

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ABSTRACT

Downregulation of LRIG1 was found in many types of cancer. However, data concerning the possible mechanism of LRIG1 reduction in cancers were not reported yet. To analyze the regulation and function of LRIG1 in colorectal cancer (CRC), 6 cell lines, 46 paired tissues from primary CRC cases were employed in this study. In CRC cell lines, under-expression of LRIG1 was correlated with promoter region hypermethylation, and restoration of LRIG1 was induced by 5-Aza-2'-deoxyazacytidine treatment. Subsequently, we ectopically expressed LRIG1 in LRIG1 low-expressing HCT-116 cells and suppressed LRIG1 in LRIG1 high-expressing LoVo cells. We found that over-expression of LRIG1 inhibits cell proliferation and colony formation and tumor growth, while knockdown of LRIG1 promotes cell proliferation and colony formation. Decreased and increased EGFR/AKT signaling pathway may partially explain the lower and higher rates of proliferation in CRC cells transfected with LRIG1 cDNA or shRNA. In clinical samples, we compared the methylation, mRNA and protein expression of LRIG1 in samples of CRC tissues. A significant increase in LRIG1 methylation was identified in CRC specimens compared to adjacent normal tissues and that it was negatively correlated with its mRNA and protein expression. In conclusion, LRIG1 is frequently methylated in human CRC and consequent mRNA and protein downregulation may contribute to tumor growth by activating EGFR/AKT signaling.

1. Introduction

Colorectal cancer (CRC) is the third most common cancer and one of the leading causes of tumor-related deaths worldwide, with an estimation of 1.2 million new diagnosed cases and more than 600,000 deaths [1,2]. In the past decades, the incidence and mortality of CRC were obviously increased [3,4], especially in China. Although the advancement of surgery and chemotherapies has been improved, the clinical end of patients with CRC still remains poor [5].

Aberrant CpG island methylation of gene promoter is a well-known mechanism in the multistep pathogenesis of CRC. Identification of novel targets silenced by promoter methylation may provide new insight into the mechanisms for the activation of tumor oncogenic pathways, or inactivation of tumor suppressive pathways, which may provide new approaches for tumor diagnostic and therapeutic improvement.

LRIG1, which are located at chromosomes 3p14 region, was suggested to be a potential tumor suppressor gene [6,7]. Increasing evidence indicates that LRIG1 inhibits the cell proliferation in prostate cancer [8], bladder cancer [9], and glioma [10,11]. Moreover, downregulation of LRIG1 was found in renal cell carcinoma [12], cervical cancer [13], oropharyngeal cancer [14]. However, the possible mechanism of downregulation of LRIG1 in tumors has not been reported yet. Therefore, our present study provides the first evidence for epigenetic regulation of LRIG1 in CRC.

In this study, we analyzed the epigenetic regulation of LRIG1 in CRC cell lines and nonmalignant colorectal tissues, and the effect of overexpression or knockdown of LRIG1 on tumor growth and EGFR/AKT signaling pathway.

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2. Materials and methods

2.1. Cell lines and samples

Five CRC cell lines (LoVo, HT-29, SW480, Caco-2 and HCT-116) were obtained from Shanghai Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences (Shanghai, China). Cells were incubated in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO_2. Normal colon epithelial cell (FHC) was cultured in DMEM medium. A total of 92 formalin-fixed and paraffin-embedded (FFPE) tissues (tumor tissues, 46; adjacent noncancerous tissues, 46) were obtained from patients who received surgical resection at the department of Oncological Surgery, the Central Hospital of Xuzhou City, from Aug 2009 to Feb 2011. All tissue specimens were examined and determined by two pathologists. Histological diagnosis of CRC based on the clinicopathological criteria defined by the Union for International Cancer Control. The study was approved by the institutional review board of the hospital and signed informed consent was obtained from participants. Clinical details are listed in Supplementary Table S1.

2.2. DNA isolation and bisulfite modification

Genomic DNA was extracted from FFPE tissues and cells using Qiagen DNeasy Tissue Kit (Qiagen, Düsseldorf, Germany). After DNA isolation, bisulfite modification was conducted using the EpiTect Bisulfite kit (Qiagen) according to the manufacturer's instructions.

2.3. Methylation specific PCR (MSP), quantitative methylation specific PCR (QMSP) and bisulfite sequencing PCR (BSP)

MSP primers were designed by using Methyl Primer Express v1.0 software (ABI, UK) and synthesized to detect methylated (M) and unmethylated (UM) alleles. Each MSP reaction contained a 100 ng of bisulfite-modified DNA, 0.2 μM of each primer, 1 μM dNTPs, 2 μl 10 × PCR buffer, and 1 unit of Taq Polymerase (Invitrogen) in a final reaction volume of 20 μl. All primers used in this study were shown in Supplementary Table S2.

Bisulfite-treated DNA 10 ng was used in QMSP with a LightCycler480 (Roche, Basel, Switzerland). Amplification reaction was carried out in 384-well plates and supplementary with 4 μM of magnesium chloride. The relative level for LRIG1 in each sample was determined as a ratio of QMSP-amplified LRIG1 to ACTB and then multiplied by 100. Each plate contained tissue samples, water blanks, and positive controls. Leukocyte DNA from a healthy people was methylated in vitro with excess SssI methyltransferase (New England Biolabs, Beverly, MA) to generate completely methylated DNA, and serial dilutions (40–0.004 ng) of this DNA were used to construct a calibration curve.

Genomic DNA from HCT-116, LoVo, HT-29, FHC cell lines, and 1 non-tumor tissue specimen was included in BSP analysis. The primers used for BSP were designed to amplify DNA spanning −421 to −125 bp upstream of the LRIG1 transcription start site and included the MSP-amplified promoter region. The PCR products were cloned into pCR2.1 vectors according to the manufacturer's protocol (Invitrogen).

2.4. 5-Aza-2′-deoxycytidine (5-Aza) treatments, RNA isolation and real-time quantitative PCR (RT-qPCR)

CRC Cells (LoVo, HT-29, SW480, and HCT-116) were treated with 5-Aza (Sigma, St Louis, MO, USA) at a concentration of 5 μM in the medium, which was renewed every 24 h for three days. Cells were harvest at 72 h and RNA was extracted.

Tissue samples and cell lines were lysed using TRI Reagent (Sigma) and total RNA was isolated. A total of 2 mg total RNA was used to synthesize first strand cDNA using the Superscript III-reverse transcriptase kit (Invitrogen). PCR reaction was conducted with 2 × SYBR Green PCR Master Mix (ABI) based on the manufacturer's protocol. ABI 7900 sequence detection system was used to amplification and the cycling condition is 95 °C 5 min, 40 cycles (95 °C for 30 s, 54 °C for 30 s and 72 °C for 30 s), 72 °C for 10 min β-Actin levels were used for normalization.

2.5. ShRNA and cDNA transfection

The shRNA sequence (sense) 5′-GCCTAAACCTGAGTTACAACA-3′ was designed to knockdown the expression of the human LRIG1 gene. The cDNA encoding the complete coding region of LRIG1 cDNA was obtained from GeneBank (NM_015541.2) and then subcloned into the lentiviral vector. Recombinant lentiviruses were produced by co-transfection 293T cells with the packaging plasmids PMD2G and PSPX2, using Lipofectamine 2000 (Life Technologies).

2.6. Western blot

Total protein extracts from five CRC cell lines and lentivirus infection cells (HCT-116 and LoVo) were obtained using RIPA lysis buffer (Sigma). Protein concentrations were measured using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Equal amounts (20 μg) of proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA) and the membranes were blocked by incubation in TBST (25 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween-20) containing 5% non-fat milk at room temperature for 1 h. Membranes were incubated with primary antibodies at 4 °C overnight followed by incubation with secondary peroxidase labeled antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.7. Cell proliferation, colony formation, and xenograft assay

After lentivirus infection, HCT-116 and LoVo cells were plated in 96-well plate at a density of 2×10^3 cells/well, and cell viability was measured at 24, 48, 72, and 96 h using the methylthiazoletetrazolium (MTT) assay kit (Promega, Madison, Wisconsin, USA). Absorbance was measured on a microplate reader (Thermo, USA) at a wavelength of 570 nm.

HCT-116 and LoVo cells were grown in six-well plates 24 h before transfection. HCT-116 Cell was transfected with empty vector or LRIG1 expression vector, while LoVo cell transfected with control shRNA or LRIG1 shRNA. Culture medium, conditioned with G418 (Invitrogen) at 100 μg/ml for HCT-116, and 500 μg/ml for LoVo was exchanged every 24 h. After 14 days, the cells were fixed with 75% ethanol for 30 min and stained with 0.2% crystal violet for 20 min and counting.

All of the mouse experiments were approved by the Animal Care Committee of the hospital. Briefly, four-week old athymic nude mice were injected into the one flank with 6×10^6 HCT116 LRIG1 overexpressed cells and the other flank with empty vector cells, so that tumor comparisons were controlled for each mouse. 21 days after injection, tumors were excised and weighed.

2.8. Immunohistochemistry (IHC) assay

Immunohisto-chemical staining of LRIG1 was performed using polyclonal rabbit antibodies against the cytosolic tails of LRIG1. The extent of staining was classified as follows: 0, <15% positive cells; 1, 15%–30%; 2, 30%–60%; 3, >60% positive cells. The intensity of

staining was scored on the following criterion: 0, no staining; 1, weak; 2, moderate; 3, strong. Cytoplasmic staining of LRIG1 was scored based on the sum of extent and intensity. A total score of 0–3 was defined to be a low expression, while a score of 4–6 was considered as high expression.

2.9. Statistical analysis

Prism 5 (GraphPad Software, San Diego, CA, USA) was used to analysis experiment data. The data were presented as the mean ± standard error of mean (SEM), and analyzed using the student's t test. LRIG1 methylation or expression levels between CRC and matched non-tumor tissues were compared using the paired-samples t test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. LRIG1 expression is down regulated by promoter hypermethylation in CRC cells

The CpG island in promoter region of LRIG1 was shown in Fig. 1A. In order to initially check if the LRIG1 gene is an epigenetic gene in CRC, we tested LRIG1 promoter methylation status in five CRC cell lines and FHC cells by using MSP. As shown in Fig. 1B, LRIG1 promoter region was fully methylated in CRC cells and partially methylated in FHC cell. Then, we randomly selected 4 paired tissue samples from CRC patients and detected the methylation status of LRIG1. We found that methylation intensity of LRIG1 was more significant in primary tumor tissues compared with adjacent non-tumor tissues.

MSP results were further validated by BSP in HT-29, HCT-116, LoVo, FHC, and 1 non-tumor tissue specimen. The promoter region (−421 to −125) including 41 CpG sites was amplified by PCR and sequenced. Representative results are shown in Fig. 1C and D. The 41 CpG sites in this region were identified to be hypermethylated in CRC cell lines, partially methylated in FHC, and almost unmethylated in nonmalignant colorectal tissue. These results indicated LRIG1 promoter hypermethylation status in CRC cells.

To demonstrate the hypothesis that downregulation of LRIG1 caused by hypermethylation of the promoter region, CRC cell lines were treated with the demethylating agent 5-Aza. The mRNA expression of LRIG1 was increased several fold in the 5-Aza treated cells compared with that in the control cells. These results strongly indicated that hypermethylation is responsible for down-expression of LRIG1 in CRC cells (Fig. 1E).

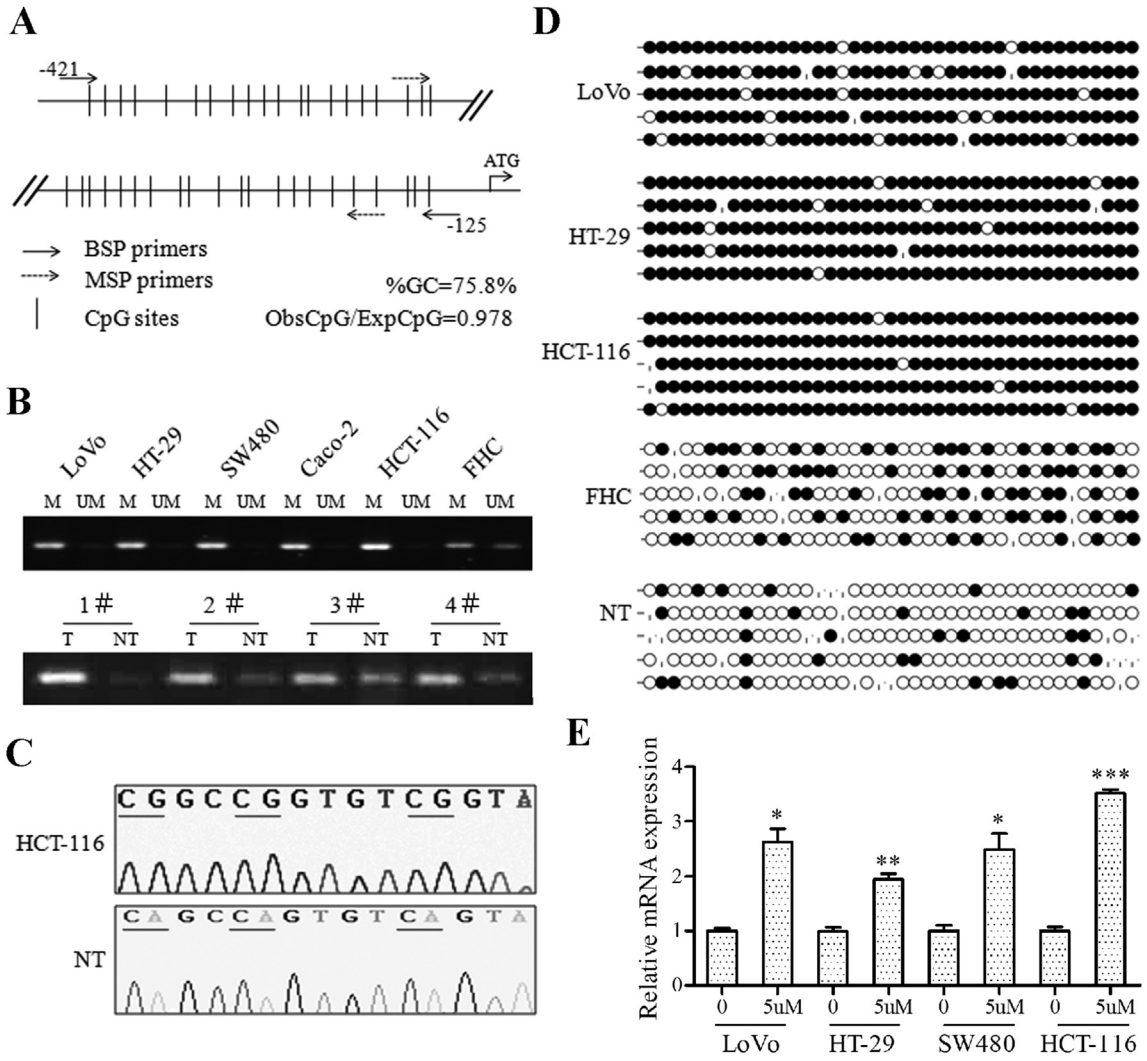

Fig. 1. Methylation analysis of LRIG1 by MSP, BSP, and 5-Aza treatment. (A) The target CpG island located in LRIG1 promoter region. (B) MSP of LRIG1 in CRC cell lines and 4 paired cancerous and non-cancerous samples. M, methylation; UM, unmethylation; T, tumor tissues; NT, non-tumor tissues. (C) Representative sequences of BSP in HCT-116 cells and non-tumor tissues. (D) Methylation status of 41 CpG sites in the promoter region of LRIG1. Black dot, methylated CG; white dot, unmethylated CG; stub, not available. (E) RT-qPCR analysis of LRIG1 mRNA expression after 0 or 5 μM 5-Aza treatment for 72 h *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; independent t-test.

3.2. Restoration of LRIG1 expression inhibits cell proliferation in vitro and in vivo

After detection of LRIG1 mRNA expression and protein level in five CRC cells (Fig. 2A and B), we decided to selected HCT-116 and LoVo cells, which had low and high levels of endogenous LRIG1, respectively, for further LRIG1 overexpression and knockdown analysis.

To determine if enhanced LRIG1 expression alters the proliferative activity of CRC cells, we generated stable HCT-116 cells that over-expressed either HA-tagged GFP cDNA (HA-GFP) or HA-tagged LRIG1 cDNA (HA-LRIG1). As shown in Fig. 2C, Cell proliferation was impaired in HCT-116 cells stably expressing HA-LRIG1 compared to control cells. The colony number is less in LRIG1 overexpression group in comparison to control group ($P < 0.01$, Fig. 2D). To confirm the inhibitory role of LRIG1 expression on cell proliferation, we knocked down LRIG1 expression in the LoVo cells by shRNA. In accordance with previous observation, we found that knockdown of LRIG1 promoted the growth of LoVo cells and colony formation (Fig. 2C and D). By in vivo analysis, when compared with control group and LRIG1 overexpressed group, we found that the xenografts generated with LRIG1 up-regulated cells had markedly smaller size and lower weight (Fig. 2E).

3.3. Effect of LRIG1 on EGFR/AKT signaling pathway

EGFR/AKT pathway has been reported to be deregulated in many tumors. To further analysis downregulation of LRIG1 modulating the observed growth promotion that might associate with downstream EGFR/AKT pathway, we examined the effect of overexpression or knockdown of LRIG1 gene on the expression of several key regulators involved in the EGFR/AKT signaling pathway. As shown in Fig. 3A, western blot analysis showed that upregulation of LRIG1 caused a significant reduction in phosphorylation of EGFR (p-EGFR) and EGFR in HCT-116 cell. The level of activated mitogen-activated protein kinase (p-MAPK), a downstream regulator of EGFR signaling, exhibited obvious decrease as well. Downregulation of p-AKT expression was also observed with LRIG1 cDNA transfection, compared with the empty vector. In contrast, knockdown of LRIG1 by shRNA displayed the opposite effect on the protein level of p-EGFR, EGFR, p-MAPK, and p-AKT, which had significant enhancement in LoVo cell (Fig. 3B). Our results indicate that LRIG1 negatively regulates EGFR/AKT signaling pathway in CRC.

To investigate whether the LRIG1 downregulation induced AKT pathway affects downstream apoptosis signaling in CRC cells, both anti-apoptotic effectors and pro-apoptotic effectors were subjected

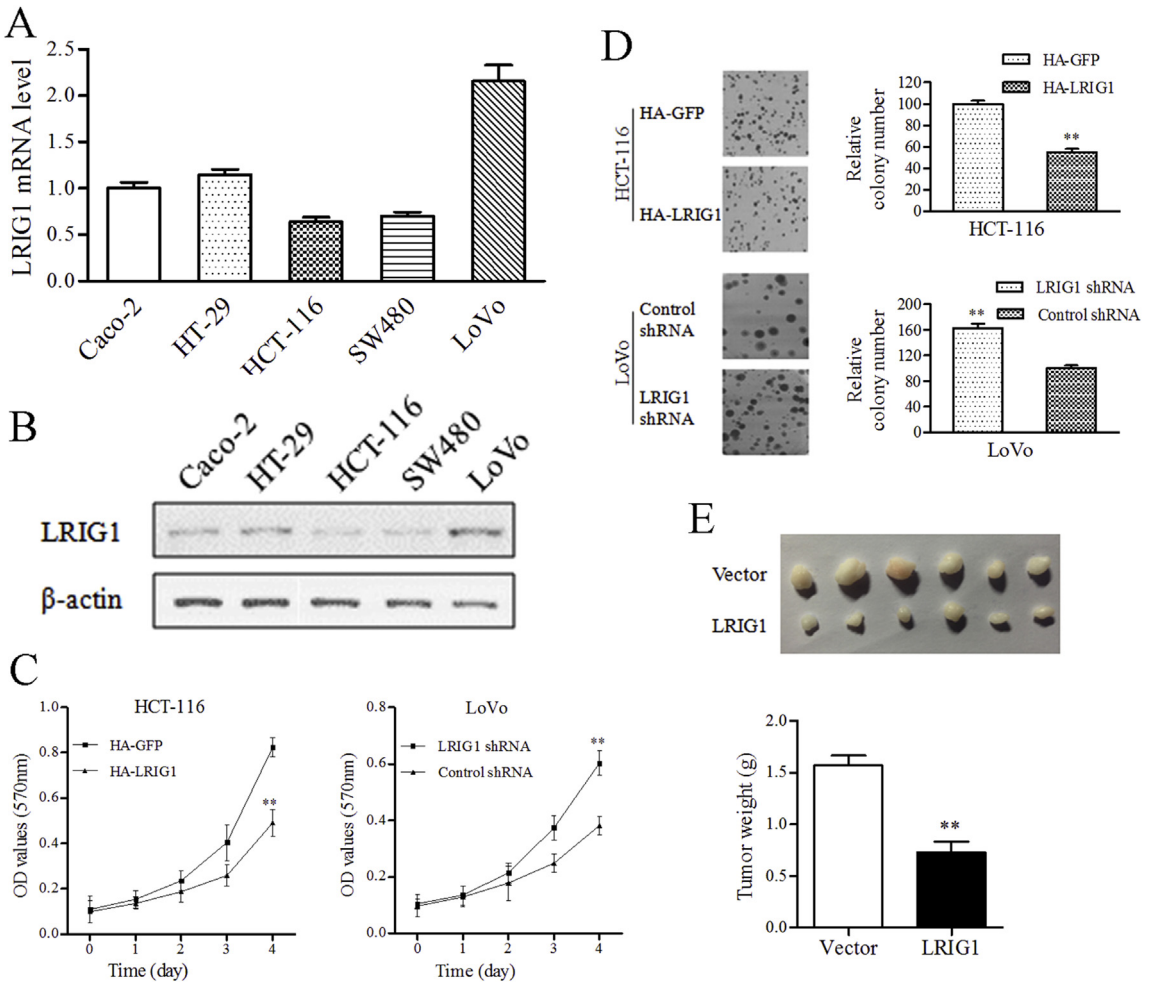

Fig. 2. LRIG1 suppresses CRC cell proliferation in vitro and in vivo. (A and B) the mRNA and protein level of endogenous LRIG1 in five CRC cell lines, respectively. (C and D) Cell proliferation and colony formation were inhibited by LRIG1 cDNA transfection in HCT-116 cells, whereas promoted by LRIG1 shRNA transfection in LoVo cells. (E) Xenograft models in nude mice were generated with transfected HCT-116 cells and xenograft tumors were weighed.

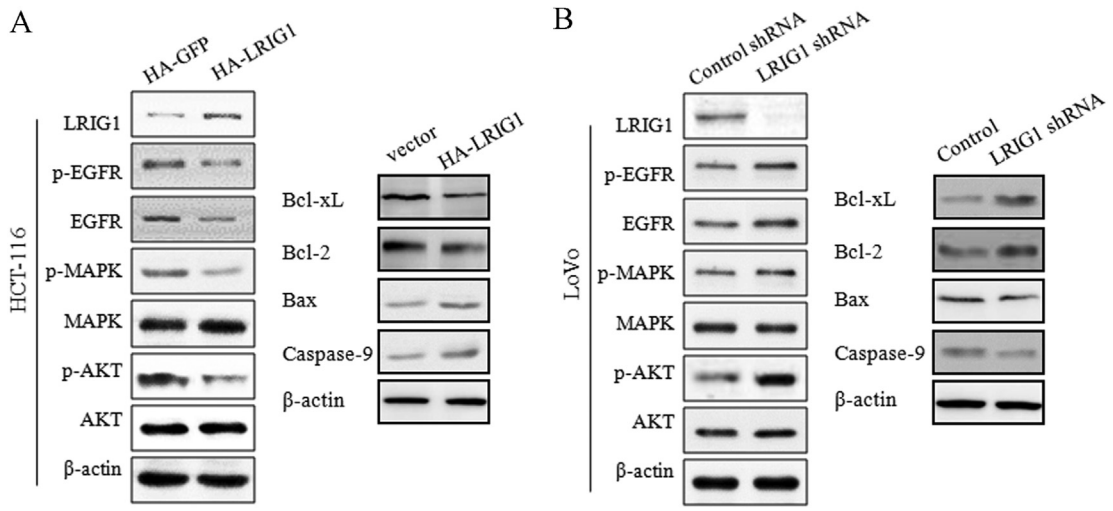

Fig. 3. Effect of LRIG1 cDNA or shRNA transfection on EGFR downstream signaling. (A) In HCT-116 cells, data showed decreased phosphorylation of EGFR and downstream MAPK, AKT and apoptosis related proteins after LRIG1 cDNA transfection. (B) In LoVo cells, data showed increased phosphorylation of EGFR and downstream MAPK, AKT and apoptosis related proteins after LRIG1 shRNA transfection.

to western blot analysis. LRIG1 inhibition resulted in an increased expression of the Bcl-2 and Bcl-xL proteins and decreased expression of Bax and caspase-9 in LoVo cells, while overexpression of LRIG1 exerted the opposite effect in HCT-116 cells (Fig. 3A and B). These results suggested that LRIG1 downregulation induced anti-apoptotic effects in CRC cells involved both pro- and anti-apoptotic factors and were partially regulated by AKT pathway.

3.4. Methylation of LRIG1 correlates with its mRNA and protein low-expression in CRC tissues

To further verify the methylation status of LRIG1 during human CRC development, we analyzed methylation level of LRIG1 in CRC tissues ($n = 46$) and adjacent normal tissues ($n = 46$), and mRNA level in 30 paired CRC tissues.

We observed a significantly increased methylation level in CRC tissues (0.037 ± 0.018) compared with non-tumor tissues (0.033 ± 0.013) ($p = 0.002$, Fig. 4A). However, it was also found that the methylation level of LRIG1 did not correlate with gender, age, clinical stage, or the overall survival of CRC patients (data not show).

The mRNA levels of LRIG1 were significantly down-regulated in cancer tissues as compared to the normal tissues (67.027 ± 10.478 vs 73.034 ± 12.920, $p = 0.001$, Fig. 4B). The expression of LRIG1 did not associate with clinicopathological features. We observed a negative correlation between LRIG1 methylation and mRNA expression (Pearson's correlation, $r = -0.502$, $p = 0.005$, Fig. 4C). These results suggest that LRIG1 expression is regulated by promoter methylation in human primary CRC.

By using IHC method, we found LRIG1 was mainly expressed in the cytoplasm in the majority of CRC cases. The immunochemical staining of LRIG1 in primary CRC tissues were shown in Fig. 4D. One sample of CRC tissue was excluded for IHC assay because of technical reason. 26 (57.8%) of the 45 primary CRC tissue samples were low for LRIG1 expression, while the other 19 (42.2%) were high. By spearman's correlation, a significantly negative correlation was found between the methylation level and protein expression of LRIG1 (Spearman $r = -0.311$; 95% confidence interval $= -0.557 \sim -0.013$; $p = 0.038$). In addition, the high expression of LRIG1 was associated with better survival ($p = 0.057$, Supplementary Fig. S1).

4. Discussion

Accumulating evidence supporting that promoter hypermethylation of tumor-related gene can be used as a sensitive marker for CRC early diagnosis, prognosis prediction, and therapeutic target [15–17]. To our knowledge, this is the first study of LRIG1 methylation in CRC.

In this study, we demonstrate that promoter methylation may play an important role in the regulation of LRIG1 expression. First of all, methylation status of LRIG1 was examined by MSP in 5 CRC cell lines, and FHC cells. LRIG1 was fully methylated in all five CRC cells, while partially methylated in FHC cells. Besides, we compared the methylation status of LRIG1 in primary tissues and adjacent normal tissues. Methylation of promoter region was more significant in tumors compared with that in non-tumor tissues. Subsequently, BSP results confirmed that LRIG1 promoter methylation was found in CRC cells, indicating a high prevalence of LRIG1 methylation in CRC. Considering epigenetic silencing of tumor-related genes has been shown to be reversible [18,19], we measured LRIG1 expression in CRC cells cultured in the presence of the demethylation agent 5-Aza. The mRNA levels of LRIG1 in CRC cells were significantly up-regulated after 5-Aza treatment. In general, these results revealed that the promoter methylation of LRIG1 has a critical role in its transcriptional downregulation, which may be involved in CRC development.

We are interest in the function of LRIG1 on tumor cell proliferation. We performed cDNA transfection in HCT-116 cell, which had low level of endogenous LRIG1, and found an inhibitory effect on cell proliferation and colony formation and tumor growth. In contrast, we also observed a stimulative effect on cell proliferation after knockdown of LRIG1 in LoVo cells. Our data was in accordance with previous studies [8–11], and expanded the knowledge of LRIG1 in CRC development.

EGFR is a well-studied, versatile signal transducer that is over-expressed in many types of tumor, including colon cancer [20,21]. EGFR regulates signals that promote cell proliferation in various types of tumor [22], and its signal transduction is modulated by stimulatory and inhibitory factors. LRIG1 was commonly reported as a negative regulator of EGFR signaling. Thus, we detected the protein level of key regulators of EGFR/AKT pathway in stably over-expressed or knockdown of LRIG1 cells. Up-regulation of LRIG1

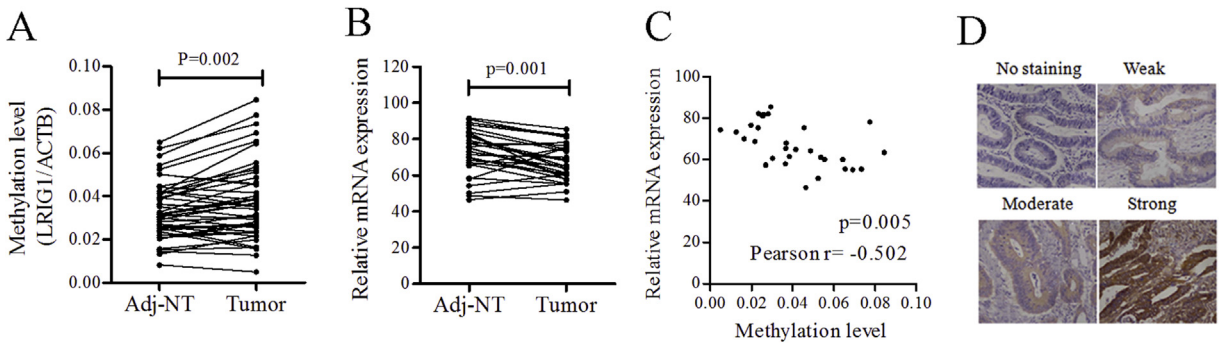

Fig. 4. The methylation, mRNA and protein expression of LRIG1 in CRC tissues. (A) Methylation level of LRIG1 was significantly increased in tumors in comparison with normal tissues, Adj-NT, adjacent-normal tissues. (B) mRNA levels of LRIG1 were decreased in tumors. (C) The negative correlation between LRIG1 methylation and mRNA expression, Pearson's correlation, $r = -0.502$, $p = 0.005$. (D) Immunohistochemistry of LRIG1 in CRC tissues.

suppresses EGFR/AKT signaling pathway, with the decreased protein level of p-EGFR, EGFR, p-MAPK, and p-AKT in HCT-116 cell, while knockdown of LRIG1 exhibited the opposite effect in LoVo cell. Our data was in consistent with previous studies [23,24]. Besides, we found a decreased or increased expression of pro- and anti-apoptotic factors after knockdown or overexpression of LRIG1 in CRC cells, suggesting LRIG1 downregulation functions as an anti-apoptotic role at least partially mediated by AKT pathway. Given LRIG1 functions as an epigenetic target in CRC and inhibits EGFR/AKT signaling, our data suggests that restoration of LRIG1 may be a potential therapeutic approach for CRC.

Clinically, as our shown, the methylation level of LRIG1 was markedly increased in primary CRC tissues, suggesting that hypermethylation of LRIG1 might be a potential biomarker for CRC diagnosis. It was also found that methylation of LRIG1 was not correlated with characteristics variables. Further analysis based on a large-scale cohort may provide more clinical significance of LRIG1 methylation. The mRNA levels of LRIG1 were significantly decreased in primary CRC tissues, which was consistent with former studies [12–14]. Furthermore, we found that the protein levels of LRIG1 were negatively associated with its methylation level, and low expression of LRIG1 protein correlated with poor prognosis, which was in accordance with former studies [25–27]. These results indicated that methylation was an important mechanism for regulating mRNA and ultimately protein levels of LRIG1 in CRC tissues.

In conclusion, this is the first study to describe LRIG1 as a novel epigenetic silencing gene in CRC, thereby providing a potential marker for early diagnosis. Moreover, our findings indicate that the promoter hypermethylation contributes to lower LRIG1 expression, which may be involved in CRC tumor growth by modulating EGFR/AKT signaling pathway.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.06.173.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.06.173.

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