Lentivirus-mediated LIGHT overexpression inhibits human colorectal carcinoma cell growth in vitro and in vivo

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Abstract. Human LIGHT (lymphotoxin-related inducible ligand that competes for glycoprotein D binding to herpesvirus entry mediator on T cells) is the 14th member of the tumor necrosis factor (TNF) superfamily and is therefore also known as TNFSF14. LIGHT has been proven to be a multifunctional molecule affecting cell proliferation, differentiation and a number of other biological processes, in particular, cell growth inhibition. However, the expression and molecular mechanisms of the LIGHT gene in human colorectal carcinoma cells remain largely unclear. In the present study, the LIGHT gene was overexpressed using a lentiviral expression vector in HCT116 human colorectal carcinoma cells in vitro and in vivo, in order to explore the mechanism by which the LIGHT gene inhibits cell growth and suppresses tumor formation. The results showed that the recombinant lentivirus with LIGHT overexpression inhibited the proliferative capacity of the HCT116 cells and significantly decreased the xenografted tumor volumes in nude mice. Furthermore, LIGHT treatment effectively initiated increased caspase-3 and decreased Bcl-2 activities in the HCT116 cells. This study provides a basis for the improved understanding of the role and molecular mechanisms of the LIGHT gene in human colorectal carcinoma cells and may facilitate further functional studies of LIGHT.

Introduction

Human LIGHT (lymphotoxin-related inducible ligand that competes for glycoprotein D binding to herpesvirus entry mediator on T cells) is the 14th member of the tumor necrosis factor (TNF) superfamily and is therefore also referred to as TNFSF14 (1). LIGHT is able to bind the lymphotoxin-β receptor (LTβR), which is expressed on numerous types of epithelial cancer, and the herpes virus entry mediator (HVEM), a receptor expressed by T lymphocytes; therefore, LIGHT is additionally known as HVEM-L (herpesvirus entry mediator-ligand) (2). LIGHT is a multifunctional molecule affecting cell proliferation, differentiation and a number of other biological processes (3). LIGHT costimulates T-cell amplification effects and enhances the cell immune reaction to tumors (4), acting as the most effective tumor immunotherapy factor (5,6). However, the mechanism by which LIGHT affects cancer cells is poorly understood.

A previous study by our group showed that LIGHT promotes HepG2 hepatic carcinoma cell apoptosis through regulating the expression of Bcl-2 and caspase-8 (7). Furthermore, LIGHT was not detected in the HCT116 colorectal cancer cell line and the overexpression of LIGHT transfected into HCT116 cells by plasmid vector inhibited cell growth. Due to the low transfection efficiency of plasmids, a stable cell line with LIGHT overexpression may facilitate further functional studies of LIGHT.

In the present study, LIGHT was overexpressed using a lentiviral expression vector in the HCT116 colorectal cancer cell line and its effects on cell biology were investigated, thus providing a basis for further study of LIGHT functions.

Materials and methods

Cell line and culture. The HCT116 human colon cancer cell line was purchased from China Centre for Type Culture Collection (Shanghai, China). The cells were cultured at 37°C in McCoy's 5α (modified) medium (Sigma, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS; Hyclone, Waltham, MA, USA) in a humidified atmosphere of 5% CO₂. The cells were detached using 0.25% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA).
Animals. Athymic nude male BALBC/c mice, weighing 17-19 g (4-5 weeks old), were purchased from the Institute of Laboratory Animal science, Chinese Academy of Medical Science (Beijing, China). The mice were maintained in specific pathogen-free, temperature-controlled isolation conditions and fed with sterilized food and autoclaved water according to the experimental animal guidelines. The use of animals in the present study complies with the Guide for the Care and Use of Laboratory Animals. All animal studies were approved by the Animal Research and Ethical Committee of Qingdao Medical College (Shandong, China).

Construction of recombinant lentivirus vector encoding the LIGHT gene. Primers for whole length cDNA of human LIGHT were designed and synthesized with sequences as follows: Sense, 5'-CGCGGATCCATGGAGGAGATGTGC-3' and antisense, 5'-CTCGTCACTCAACCATGAAAGCCCC-3'. Briefly, the LIGHT gene was amplified by HotStar Taq DNA polymerase (Qiagen, Hilden, Germany) using the pAAV-LIGHT plasmid as a template and then the LIGHT gene and pLenti vector were cut by SalI and BamHI enzymes. Following recycling electrophoresis, the LIGHT gene was subcloned into the pLenti plasmid and the recombinant plasmid, pLenti-LIGHT, was identified by incision with the SalI and BamHI enzymes and sequencing. Primer synthesis and DNA sequencing were performed by Shanghai Shangon Co. Ltd. (Shanghai, China). The viral particles were generated by the cotransfection of 293T cells (ATCC) via a calcium phosphate-mediated transfection method with pLenti-LIGHT or pLenti-GFP and three packaging vectors. Three days after transfection, the cell culture supernatants were harvested (1,600 x g for 5 min), filtered through 0.45-µm pore size filters, and concentrated 100-fold by ultracentrifugation at 7,000 x g (1,600 x g for 5 min), stored in small aliquots, and then used for infection.

Transfection and selection of stable HCT116 cell lines (HCT116/LIGHT). For the transfection of the tumor cell lines, lentiviral vectors harboring LIGHT were constructed and the HCT116 cells were infected. Briefly, the HCT116 cells were cultured in McCoy's 5a medium containing 10% FBS and when they reached the exponential growth phase, 1.0x10³ cells per well were plated in 24 plates. Next, 300 µl complete culture medium, containing recombinant lentiviruses, control lentiviruses or McCoy's 5a medium (all containing 6 µg/ml polybrene; Sigma) was added into the plates when the cells reached 50-60% confluence. Two days later, the virus-containing medium was replaced with fresh complete medium. The expression level of GFP was observed under a microscope after 3 days. Medium containing blasticidin (6 µg/ml; Merck KGaA, Darmstadt, Germany) was added every 3 or 4 days to screen the stable infected cell lines (HCT116/LIGHT or HCT116/GFP, with clear clone formation) until the uninfected cells were almost completely removed.

Determination of the optimal multiplicity of infection (MOI). To assess the efficiency of lentiviral transduction in the human HCT116 cells, the cells were infected with pLenti-GFP at various MOIs for 24 h. The supernatant was then changed to fresh complete medium every other day. After 72 h, GFP-expressing cells were detected by fluorescence microscopy (Olympus; Tokyo, Japan).

Semi-quantitative reverse transcription-PCR analysis. The total RNA of HCT116/LIGHT, HCT116/GFP or the control cells was extracted using RNAiso reagent (Takara, Japan), and then converted into cDNA using a PrimeScript™ RT reagent kit (Takara), according to the manufacturer's instructions. The specific oligonucleotide primers of the LIGHT (PCR product 128 bp) and GAPDH (PCR product 151 bp) genes were as follows: Sense, 5'-GTACGGCCCTCAATGTTTGTG-3' and antisense, 5'-CCCATACGGCAACAGCAAGAGA-3'; and sense, 5'-CTTACACCCCCGAGAG-3' and antisense, 5'-GATGTTCGAGAGCCCG-3', respectively. The reaction conditions of pre-denaturation were 95°C for 3 min, 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec, with 22 cycles for GAPDH and 29 cycles for LIGHT (the cycles were based on PCR kinetics) and a total reaction volume of 20 µl. Each PCR was repeated three times. The products were subsequently analyzed using 2% agarose gel electrophoresis. The semiquantitative analysis of LIGHT and GAPDH mRNA levels was measured using the Syngene Gel Imaging System and analysis software (Syngene Co., Cambridge, UK).

Expression of LIGHT protein by ELISA. Cell supernatants were collected after 48 h and the expression of LIGHT protein was detected by ELISA according to the manufacturer’s instructions (R&D, Minneapolis, MN, USA). The kit was capable of detecting LIGHT with a minimal detectable dose as low as 10 pg/ml. The primary wavelength was 450 nm (optionally 620 nm as the reference wavelength). All the tests were repeated three times with three wells per group.

Western blot analysis. The HCT116 cells were plated onto type I collagen-coated 25-cm² flasks, then treated with Lenti-GFP or Lenti-LIGHT for 48 h in basal medium containing 10% FBS. The HCT116/LIGHT, HCT116/GFP or control cells were harvested with a cell scraper, and stored at -80°C until protein extraction. The pellets were resuspended with a lysis buffer [50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 100 µM 4-aminophenylmethanesulfonyl fluoride, 1 µg/ml aprotinin, 5 µg/ml leupeptin, 1 µg/ml pepstatin A and 50 µg/ml antipain] and then mixed well at 4°C. Following centrifugation, the protein concentration of each supernatant was determined using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA). Samples were subjected to 10% SDS-PAGE and subsequently transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA) in a transfer buffer. These membranes were blocked in BlockAce (Dainippon Seiyaku, Japan) overnight at 4°C. Rabbit anti-caspase-3,-Bel-2 or -GAPDH antibodies (Abcam, Cambridge, MA, USA) were used as primary antibodies at a 1:1,000 dilution in 10% BlockAce for 30 min. The samples were then washed in phosphate-buffered saline containing 0.05% Tween-20 (Bio-Rad). Goat anti-rabbit IgG was used as secondary antibody at a 1:5,000 dilution for 30 min. An enhanced chemiluminescence (ECL) detection system (Pierce Biotechnology Inc., Rockford, IL, USA) was then used to

928 WANG et al.: LIGHT OVEREXPRESSION INHIBITS HCT116 CELL PROLIFERATION
visualize immunoreactive protein complexes. An autoradiograph was obtained and the protein levels were measured using a FluorS scanner and Quantity One software for analysis (Bio-Rad).

Analysis of cell proliferation (8). The cells (1.0x10^3/well) were plated and treated in 96-well plates (three wells per group, total 5 plates) for 24, 48, 72, 96 and 120 h, respectively. At the indicated times, the medium was removed and fresh medium containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 0.5 mg/ml; Sigma) was added to each well. The cells were incubated at 37˚C for 4 h and then the medium was removed and 150 µl solubilization solution (DMSO) was added and mixed thoroughly. Absorbance from the plates was read on a Safire II spectrometer reader (Tecan, Männedorf, Swiss) at 490 nm (8).

Subcutaneous human colorectal cancer cell xenograft growth and oncogenicity. Balb/c nude mice were maintained in specific pathogen-free, temperature-controlled isolation conditions, and fed with sterilized food and autoclaved water. Subsequent to being washed with serum-free McCoy’s 5α medium, the cells were collected and 200 µl cell solution (1.0x10^7 cells in 200 µl PBS, with a viability of >95%) was injected subcutaneously into the right-side of the backs of the mice. The sizes of the transplanted tumor xenograft were measured periodically until the long diameter of tumor xenograft was >1 cm. The tumor xenografts were then dissected and measured with a vernier caliper. The tumor volumes were calculated using the following formula: Tumor volume = long diameter x short diameter^2 / 2).

Statistical analysis. All data were shown as the mean ± SD unless otherwise mentioned. Statistical analyses were performed using SPSS 11.5 (SPSS Inc., Chicago, IL, USA). Differences were assessed between the two groups using a t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Identification of recombinant lentiviral vectors and GFP visualization. The 1.5% agarose gel electrophoresis showed a LIGHT gene product, recombinant plasmid and pLenti plasmid (plasmids were incised by SalI and BamHI), as shown in Fig. 1. The PCR product of the LIGHT gene was ~750 bp and the recombinant plasmid exhibited 6.6 kb and 750 bp products after enzyme incision. The sequence of the LIGHT gene was demonstrated to be the same compared with the sequence from GenBank (Fig. 1).
The HCT116 cells exhibited a high lentiviral transduction efficiency at a MOI of 5 at 72 h. The visualized GFP results showed that the number of GFP-expressing cells increased in an MOI-dependent manner (Fig. 2). As an MOI of 5 was shown to be optimal for cell infection, an MOI of 5 was selected for subsequent studies. In total, ~90-95% of cells in the HCT116/LIGHT and HCT116/GFP cell lines infected with lentivirus expressed significant fluorescent signals.

The number of PCR cycles required for LIGHT and GAPDH was 29 and 22, respectively, as determined by the amplification dynamic experiments (data not shown). The lengths of the PCR products were 128 bp (LIGHT) and 151 bp (GAPDH), respectively. The PCR product electrophoresis and semiquantitative analysis showed that the relative mRNA level of LIGHT in the HCT116/LIGHT cell line was significantly higher than that of the blank control (~5.42 times more than that of the blank control) and HCT116/GFP cells (Fig. 3).

Overexpression of LIGHT protein. The A450 values in the HCT116/GFP and blank control groups showed no difference with the use of McCoy’s 5α complete medium, which indicated that endogenous LIGHT in the HCT116 cells was not expressed or was weakly expressed (<10 pg/ml). The protein levels of LIGHT in the HCT116/LIGHT cells were significantly increased compared with the HCT116/GFP and blank control groups (3.16±0.15 vs. 0 ng/ml).

**LIGHT activates caspase-3 and inhibits Bcl-2 activation in HCT116 cells.** Several caspases play significant roles in TNF family-mediated apoptosis. To evaluate the anti-apoptotic effect of LIGHT on the HCT116 cells, a western blot analysis was performed to investigate the processing of caspase-3 using cell lysates from the HCT116 cells pretreated with or without LIGHT. At 48 h after Lenti-GFP or Lenti-LIGHT incuba-
tumor T-cell immu due to the incomplete elimination of Bcl-2 remained as
modulate or reinforce one and/or multiple immune cell func
development of cancer. The cytokine therapy of tumors may
an infection is cleared or will persist to pose a risk for the
or kill tumor cells. Immune responses markedly affect whether
and cells. The immunotherapy of tumors may stimulate and
growth frequently occurs 
chemotherapy and radiotherapy, tumor recurrence or metas
clinical incidence (9). Despite surgical resection, systemic
malignant tumor with a high
Colorectal cancer is a type of malignant tumor with a high
Discussion
Colorectal cancer is a type of malignant tumor with a high
imaging, the cleavage product of caspase-3 was clearly detected,
indicating that caspase-3 activation had occurred by that
time (Fig. 4). Furthermore, lower levels of Bcl-2 remained as
compared with the untreated control. There was no differ-
ence in the quantity of cleavage products or Bcl-2 observed in
the reactions with Lenti-GFP and the untreated control.
Effect of LIGHT on HCT116 cell proliferation. Growth curves
showed a slight deceleration of cell growth in the HCT116/GFP
group compared with the blank control group. There was
significant growth inhibition in the HCT116/LIGHT group
compared with other groups, which indicated that the expres-
sion of LIGHT caused inhibition of cell growth (Fig. 5).
Effect of LIGHT on oncogenicity in nude mice. Eight days
after subcutaneous cell injection, the tumor xenografts were
successfully inoculated and the volumes of the tumor xeno-
grafts became enlarged with the progression of time. The
tumor volume in the HCT116/LIGHT group was smaller than
in the HCT116/GFP and blank control groups (3.08 times in
the blank control group and 2.80 times in the HCT116/GFP
group as compared with that in the HCT116/LIGHT group; Fig. 6).

Discussion
Colorectal cancer is a type of malignant tumor with a high
clinical incidence (9). Despite surgical resection, systemic
chemotherapy and radiotherapy, tumor recurrence or metas-
tasis frequently occurs due to the incomplete elimination of
tumor cells and the inadvertent impairment of normal tissue
and cells. The immunotherapy of tumors may stimulate and
reinforce the immune system of the body and thus control and/
or kill tumor cells. Immune responses markedly affect whether
an infection is cleared or will persist to pose a risk for the
development of cancer. The cytokine therapy of tumors may
modulate or reinforce one and/or multiple immune cell func-
tions after cytokine injection (10,11). Cancer immunotherapies
employing the tumor necrosis factor superfamily (TNFSF)
molecules exhibit antitumor effects through two predominant
mechanisms, the direct killing of tumor cells and indirect
killing by activating antitumor immunity. The former mecha-
nism is limited to tumors that express the appropriate tumor
necrosis factor receptor superfamily (TNFRSF) molecules,
while the latter works irrespective of tumor type, so it may
have broad applicability as a cancer therapy (12).
LIGHT is a lymphotoxin analogue of glycoprotein D
binding to HVEM on T cells, which may be expressed on acti-
vated T cells and premature dendritic cells. LIGHT interacts
with two distinct cell-membrane receptors, HVEM and LTβR,
and one decoy receptor, Tr6/DcR3 (1,13,14). LIGHT may
also induce the apoptosis of tumor cells expressing LIGHT
receptors (6) and synergistically induce tumor cell apoptosis
with IFN-γ (7,17). Furthermore, LIGHT may induce the
expression of Mig and IP-10, chemotactic factors in antitumor
angiogenesis, inhibit tumor angiogenesis and act with natural
killer (NK) cells (18) and accelerate antitumor T-cell immu-
nity, which may result in delayed growth or the spontaneous
regression of tumors (4), all indicating that LIGHT may be an
significant antitumor factor.

The ideal viral vector should provide efficient gene trans-
fers, stable long-term gene expression and good biological
safety. The lentivirus systems used in the present study are the
third generation of lentiviral vectors and HIV-based expres-
sion vectors, which offer unique versatility and robustness as
vehicles for gene delivery (19). The lentivirus vectors offer
significant advantages over retroviral vectors in the process of
gene delivery to target cells (20,21), they are genetically altered
from wild-type HIV so as to increase their biosafety and they
may transduce a wide range of cell types and integrate into the
host genome in dividing and post-mitotic cells, resulting in
long-term expression of the transgene in vitro and in vivo (22).

In the present study, a lentivirus coexpressing GFP and
LIGHT genes was first constructed. GFP was used as a
reporter gene to monitor the expression of LIGHT. LIGHT
was overexpressed in the HCT116 colorectal cancer cell line
using pLenti-LIGHT and then the stable and overexpressed
LIGHT mRNA and protein cell lines (HCT116/LIGHT) were
screened. The results indicated that the mRNA and protein
levels of LIGHT in the HCT116/LIGHT cells were higher than
the levels in the HCT116 cells, while the overexpression of
LIGHT clearly inhibited the growth of the HCT116 cells and
the oncogenicity of the cells in the nude mice. Furthermore,
the present study demonstrated that LIGHT may inhibit the
cellular proliferative capacity of the HCT116 cells via the
upregulation of caspase-3 and the downregulation of Bcl-2.

The effective immunotherapy of tumors not only inhibits
or eliminates the local tumor burden, it also induces certain
types of protective immunity in the body and thus may prevent
further recurrence of tumor cells. Therefore, LIGHT may serve as a promising tumor immunotherapy factor and its mechanism of action consequently requires further investigation.

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