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Underlying Mechanisms for LTF Inactivation and its Functional Analysis in Nasopharyngeal Carcinoma Cell Lines

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ABSTRACT

The lactoferrin (*LTF*) gene, located at 3p21.3, behaves like a tumor suppressor gene in diverse tumors. To elucidate the exact role of *LTF* in NPC, we first detected its expression level in seven NPC cell lines by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). The results showed the mRNA level of *LTF* was nearly undetectable in all the seven NPC cell lines, while it could be detected in chronic nasopharyngitis tissues. Subsequently, we used methylation-specific PCR (MSP), microsatellite assay, PCR-single-strand conformation polymorphism (PCR-SSCP) and sequencing methods to examine the promoter methylation, loss of heterozygosity (LOH) and gene mutation of *LTF* in NPC cell lines respectively. Consequently, we found that 100% (7 of 7) of NPC cell lines were methylated in *LTF* promoter, only one cell line (14%, 1 of 7) had LOH and gene mutation of *LTF*, respectively, while *LTF* exhibited re-expression in all cell lines after 5-aza-dC treatment, indicating promoter methylation should be the key mechanism causing *LTF* downregulation in NPC cell lines. Furthermore, patched methylation assay confirmed that promoter methylation could down-regulate *LTF* gene expression in NPC cells. Finally, we investigated the function of *LTF* in NPC cell lines by gene transfection. Restoration of *LTF* expression in NPC cells resulted in blockage of cell cycle progression, significant inhibition of cell growth and a reduced colony-formation capacity in vitro and obviously weaker tumor formation potential in vivo. In conclusion, our data indicate *LTF* may participate in NPC carcinogenesis as a negative effector, that is, a tumor suppressor gene. J. Cell. Biochem. 112: 1832–1843, 2011.

KEY WORDS: LACTOFERRIN; NASOPHARYNGEAL CARCINOMA; HYPERMETHYLATION; INACTIVATION; TRANSFECTION; GENE FUNCTION; TUMOR SUPPRESSOR GENE; GENE MUTATION

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Nasopharyngeal carcinoma (NPC), a kind of epithelial malignancy with high incidence in Southeast Asia and Southern China, shows a remarkable ethnic susceptibility and familial aggregation. The tumorigenesis of NPC is a multi-step process involving various factors, including Epstein–Barr virus infection, accumulation of epigenetic and genetic alterations, dietary and environmental factors and so on. Until now relatively little is known about the exact molecular changes associated with NPC. Studies of allelic imbalance [Chen et al., 1999; Hui et al., 1999; Fang et al., 2001] and suppression of tumorigenicity [Cheng et al., 1998] have consistently suggested that the short arm of chromosome 3 (3p) harbors tumor suppressor genes (TSGs), whose inactivation should play important roles in the development of NPC. In addition, linkage analysis in familial NPC also indicates that the susceptibility locus is mapped on 3p21 [Xiong et al., 2004].

The lactoferrin gene (*LTF*, also referred to as lactotransferrin gene), located at human chromosomal 3p21.3 region, is a member of the transferrin gene family. It encodes an 80-kDa glycoprotein which is the major iron-binding protein in breast milk and body secretions with an antimicrobial activity, making it become an important component of human innate immune system. LTF protein demonstrates a broad spectrum of properties, including regulation of iron homeostasis, anti-inflammatory activity, regulation of cellular growth, and differentiation [Ward et al., 2005]. A number of reports have suggested that LTF has an anti-tumorigenic role through a variety of mechanisms such as regulation of NK cell activity, chemopreventive activity, modulation of G_1 protein expression, inhibition of cell proliferation and enhancement of apoptosis [Artym, 2006]. LTF has an anti-tumor function and can inhibit development and metastasis of tumors in animal models [Bezault et al., 1994; Iigo et al., 1999]. Some studies have shown that *LTF* expression is down-regulated in human lung cancer [Finkbeiner et al., 1993; Iijima et al., 2006], breast cancer [Penco et al., 1999], gastric cancer [Lee et al., 2003], glioblastoma [Tuccari et al., 1999], prostate cancer [Shaheduzzaman et al., 2007], and NPC [Yi et al., 2006], etc. It has been found that genetic (LOH, gene mutation) and epigenetic (promoter hypermethylation, histone deacetylation) alterations are responsible for aberrant expression of *LTF* gene in these malignancies.

In this study, we further evaluated *LTF* expression in NPC cell lines as well as analyzed the molecular mechanisms underlying aberrant expression of *LTF* in these NPC cell lines. Furthermore, we investigated the functions of *LTF* in NPC cells by restoration of its expression.

MATERIALS AND METHODS

TISSUES, BLOOD SAMPLES, AND CELL LINES

Eight chronic nasopharyngitis (NP) biopsies and three peripheral blood samples of NPC patients were obtained with patients' consent at Hunan Cancer Hospital (Changsha, China). All the NP biopsies were reviewed by an otorhinolaryngologic pathologist, performed pathological detection and proved to be tissues containing intact nasopharyngeal epithelia with mild inflammation. All the fresh tissues were snap-frozen in liquid nitrogen and stored until required. The present study was approved ethically by the institutional review board at Central South University. All patients provided informed written consent.

The NPC cell lines used in present study included the followings: HNE1, HNE2, HNE3, CNE1, CNE2, 5-8F, and 6-10B. All the NPC cell lines were propagated in RPMI-1640 (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum at 37°C in a humidified 5% CO_2 atmosphere.

DNA/RNA EXTRACTION

Genomic DNA from NPC cell lines and peripheral blood lymphocytes of NPC patients was isolated using Universal Genomic DNA Extraction Kit (TaKaRa, Shiga, Japan), and total RNA was prepared from NPC cell lines and fresh frozen NP tissues with TRIzol Reagent (Invitrogen, Carlsbad), in accordance with the manufacturer's instructions.

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR) AND REAL-TIME QUANTITATIVE PCR (QPCR)

Semi-quantitative RT-PCR was performed to detect the mRNA expression level of *LTF* in NPC cell lines and NP tissues. cDNA was synthesized from total RNA (2 μg) using oligo(dT) as the primer with a commercially available reverse transcription system (Promega, Madison) according to the manufacturer's protocol. Then the reaction of PCR was carried out to amplify the 207 bp region of *LTF* using primers as previously described [Yi et al., 2006]. Meanwhile, glyceraldehyde phosphate dehydrogenase (*GAPDH*) was amplified as an internal control. One fifth of RT-PCR products were separated through 1.0% agarose gel containing ethidium bromide. The sizes of the RT-PCR products were 207 bp for *LTF* and 498 bp for *GAPDH*. The intensity of each band was measured by Image Master VDS (Pharmacia Biotech, Piscataway), and analyzed by VDS software version 2.0 for band quantification. The expression levels of *LTF* in NPC cell lines and NP tissues were investigated after they were normalized by transforming them into two groups of ratios of the band intensity of *LTF* over that of *GAPDH* of the same sample.

The synthesized cDNA was also used for qPCR with SYBR Green I PCR kit (BioWhittaker, Walkersville, MD) to quantitatively analyze *LTF* mRNA expression levels in NPC cell lines and NP tissues. The primers for *LTF* and *GAPDH* were identical with those for RT-PCR. The protocol was also as same as that previously designed by us [Yi et al., 2006].

ALLELIC LOSS ANALYSIS

To examine the loss of heterozygosity (LOH) in the *LTF* locus, five microsatellite markers on chromosome 3p21.3 were selected, which encompassed the *LTF* gene. Primers and PCR parameters for amplification of microsatellite markers D3S4169, GBD:181215, D3S1478, G59627, and RH119558 are available through the genome database on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov). The microsatellite markers were amplified by PCR from 50 ng DNA extracted from seven NPC cell lines and three blood samples of NPC patients which were used as controls.

After amplification, 6–8 μl of the reaction mixture was mixed with 8 μl of loading dye (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol), heat denatured,

chilled on ice, then electrophoresed on a 6% polyacrylamide gel containing 8 M urea. The DNA bands were visualized by silver staining. LOH was scored if one of the heterozygous alleles showed at least a 50% reduction in intensity of NPC cell line DNA as compared with the blood DNA.

MUTATION ANALYSIS

Polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) were performed for screening the mutations in the promoter region (−476 to −275 bp and −305 to −50 bp), exon 1 and exon 2 of *LTF* gene using extracted genomic DNA from seven NPC cell lines and three blood DNA of NPC patients. The PCR products of NPC cell lines whose PCR-SSCP pattern showed difference from blood DNA were sequenced to verify the types of mutations.

METHYLATION ANALYSIS

To investigate the methylation status of *LTF* promoter region (−53 to +112 bp) enriched with CpG sites, methylation-specific PCR (MSP) was carried out. The MSP products of NPC cell lines were sequenced to confirm the methylation status. The primer sequences and the detailed method for PCR-SSCP and MSP were described in our published report [Peng et al., 2006; Yi et al., 2006].

The cell lines were seeded at 3×10^5 cells/ml in their culture media and treated with 50 μM 5-aza-dC (Sigma) for 96 h. The media containing drugs were replaced every 24 h. As a control, cell lines were mock-treated in parallel with the addition of an equal volume of PBS without the drug. We prepared total genomic DNA and RNA using Universal Genomic DNA Extraction Kit (TaKaRa) and TRIzol Reagent (Invitrogen). Then MSP of the gDNA and RT-PCR analysis of the RNA were performed according to the manufacturer's protocols.

REGIONAL OR PATCH METHYLATION ANALYSIS

To further elucidate whether the methylation of *LTF* promoter can cause its downregulation, we performed the promoter regional methylation assay. Briefly, different promoter fragments were amplified by PCR using the normal human blood genomic DNA as the template, methylated in vitro with CpG methylase (*Sss* I), ligated into the pGL3-basic luciferase reporter plasmid and the recombinants were respectively named pGL3B/P1-116^m, pGL3B/P1-400^m, pGL3B/P1-615^m, pGL3B/P1-940^m, and pGL3B/P2-328^m, according to the length of *LTF* promoter fragments. For comparison, unmethylated *LTF* promoter fragments without treatment with *Sss* I were also cloned into pGL3-basic vectors and the recombinants were named pGL3B/P1-116^c, pGL3B/P1-400^c, pGL3B/P1-615^c, pGL3B/P1-940^c, and pGL3B/P2-328^c, accordingly. To ensure thorough methylation of the *LTF* promoter fragments by *Sss* I, methylation-sensitive *Ssi* I was used. All the methylated and unmethylated luciferase reporter recombinants were sequenced and transfected into CNE2 cells, separately. CNE2 cells were plated in a six-well plate at a density of 2×10^5 cells/well. After 24 h, different methylated and unmethylated *LTF* promoter fragments-contained luciferase reporter plasmids were introduced into CNE2 cells by Lipofectamine 2000 reagent (Invitrogen). Another 48 h later, cells were washed three times, suspended in 200 μl 1 × passive lysis buffer (Promega), and firefly luciferase activities were measured by the Dual-Luciferase reporter assay system (Promega) and a GloMax 20/20 luminometer (Promega), according to the manufacturer's protocol. The Renilla luciferase plasmid pRL-SV40 (Promega) was co-transfected to standardize transfection efficiency in each experiment.

CONSTRUCTION OF LTF EUKARYOTIC EXPRESSION VECTOR

The *LTF* full-length cDNA cut from pCMV6-XL5-LTF vector (Origene, Rockville, MD) was cloned into the *Not* I site of pcDNA3.1(−) vector (Invitrogen), resulting in *LTF* eukaryotic expression vector (pcLTF) which was further identified by sequencing.

TRANSFECTION

CNE2 cells were seeded into six-well plates with RPMI-1640 medium at a density of 1×10^5 cells/well so that the confluence would reach approximately 70–80% at the time of transfection. Stable transfection was performed using Lipofectamine™ 2000 reagent (Invitrogen) following the manufacturers' instructions. G418 (600 μg/ml) was used to select the clones stably expressing *LTF*. RT-PCR and Western blotting were performed to detect *LTF* expression at mRNA and protein levels in the G418-resistant cell clones, respectively. As a control, the blank plasmid, pcDNA3.1(−), was also transfected to CNE2. All the following experiments were conducted in parallel on *LTF*-transfected CNE2 cells (named CNE2-LTF) and blank vector-transfected CNE2 cells (named CNE2-pc3.1) which were negative for *LTF* expression.

WESTERN BLOTTING

Cell lysates were prepared using commercial cell lysis buffer (Pierce Biotech, Rockford, IL). Proteins (60–80 μg) from whole cell lysates were separated by SDS–PAGE and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). The membrane were blocked with 5% non-fat milk in TBST (1× Tris buffered saline, 0.1% Tween-20) for 2 h. The primary antibodies in this assay were a 1:300 dilution of rabbit polyclonal to lactoferrin (Abcam, Cambridge, UK) and a 1:600 dilution of mouse monoclonal anti-α-tubulin (Santa Cruz Biotech, CA) which was used as a loading control. The secondary antibodies were a 1:2,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG antibody (Santa Cruz Biotech). Finally ECL Western blotting detection system (Pierce Biotech) and X-ray films were used to develop the blot images.

IMMUNOCYTOCHEMICAL STAINING

Cultured CNE2-LTF cells and human tumor xenograft tissue slides were immunostained with antibody for LTF using the immunocytochemical protocol described by Van and Brinkley [1999] to assess the intracellular localization and expression levels of LTF protein. Briefly, cells were washed in PBS, fixed with 4% paraformaldehyde in PBS for 20 min, and treated with 0.5% Triton X-100 in PBS (PBS-T) for 30 min. Tumor tissue sections were dewaxed in xylene, rehydrated in graded alcohols, and placed in dH_2O. Subsequently, endogenous peroxidase was inactivated by incubation in 3% H_2O_2 in dH_2O for 30 min at room temperature (RT). After rinsed with PBS-T, sections were treated in 5% bovine serum albumin (BSA) in PBS-T

(2 h, RT). Then rabbit polyclonal antibody to LTF, Histostain^{TM}-plus kit (Zymed, USA) and diaminobenzidine (DAB) substrate kit were used to detect LTF protein. Finally, the slides were lightly counterstained with hematoxylin, dehydrated, mounted with Neutral Balsam (Shanghai Specimen and Model Factory, Shanghai, China) and photographed under a microscope. For a negative control, the primary LTF antibody was replaced with PBS.

FLOW CYTOMETRY (FCM)

Cell cycle profiles were analyzed by FCM. Cells (1×10^6) were collected and washed with PBS, fixed in ice-cold 70% ethanol and stored at 4°C. After resuspended in PBS containing 100 μg/ml RNase A and 20 μg/ml propidium iodide (PI) for 30 min, samples were analyzed by flow cytometry (FACS Calibur, BD, USA). The cell cycle phase distribution was calculated from the resultant DNA histogram using Mod Fit LT software.

MTT

MTT assay was performed to assess the effect of LTF on cell proliferation. Cells were inoculated in 96-well plates with a density of 2×10^3 cells/well in volume of 200 μl. Three wells had no cells, serving as a control for the minimum absorbance. Cells were propagated at 37°C in a humidified 5% CO_2 atmosphere. The culture medium was discarded after 24 h of grouping, and 150 μl/well MTT (0.5 mg/ml) solution was supplemented for 4 h at 37°C followed by addition of 150 μl/well DMSO. Absorbance value was measured at 490 nm using a model 550 microplate reader (ELx800, Bio-Tek, USA), and then growth curves were drawn using EXCEL software.

COLONY FORMATION ASSAY

Cells (1×10^3) were seeded in six-well plates. After incubation of 8 days at 37°C in a 5% CO_2 incubator, the cells were fixed with methanol and stained with 0.4% crystal violet. Colonies containing at least 50 cells were counted under inverse microscope. Colony formation ratio (%) = cell colony amounts/1,000 × 100%.

TRANSPLANTATION INTO NUDE MICE

CNE2-LTF cells and CNE2-pc3.1 cells (5×10^6/each type) were injected *s.c.* into three 4-week-old male nude mice and three 4-week-old female nude mice (Shanghai Slac Laboratory Animal Co., Ltd, Shanghai, China). All the mice were killed 4–6 weeks after injection and examined for tumors. Mice were treated according to the guidelines of the Central South University Animal Committee. Tumor tissue samples were collected and fixed by immersion in 10% buffered formalin. After fixation for 24 h, tissues were dehydrated, paraffin embedded, sectioned at 4 μm, and stained with haematoxylin and eosin (HE) for histological examination. Paraffin sections were also placed in polylysine-coated slides to perform the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method and LTF immunohistochemistry.

EVALUATION OF APOPTOSIS BY TUNEL METHOD

For in situ detection of DNA fragmentation in paraffin-embedded tumor tissue sections, the TUNEL method was performed using In Situ Cell Death Detection Kit, POD (Roche, Basel, Switzerland), following manufacturer's instructions. Briefly, tumor tissue sections were deparaffinized and rehydrated. Protein digestion was done by incubating tumor tissue sections in 20 μg/ml proteinase K (Invitrogen) for 15 min at RT. The labeling mixture containing fluorescein-labeled dUTP in TdT enzyme buffer (TUNEL Reaction Mixture) was added to sections and incubated at 37°C in an humidified chamber for 1 h. Sections were rinsed with PBS, covered with 50 μl converter-POD and incubated for 30 min at RT. Then, sections were rinsed with PBS and incubated with DAB substrate until color development achieved. Finally, sections were washed, counterstained in haematoxylin, dehydrated, and mounted with Neutral Balsam. As a negative control, active TUNEL reaction mixture was replaced by fluorescein-labeled dUTP.

STATISTICAL ANALYSIS

All the experiments were carried out in triplicate. Difference of *LTF* expression between NPC cell lines and NP tissues was examined by Wilcoxon rank sum test. Differences between mean values were assessed by two-tailed *t*-test. All statistical analysis was performed using SPSS version 10.0 statistical software for Windows (SPSS, Chicago). A *P*-value <0.05 was considered to be statistically significant.

RESULTS

EXPRESSION OF LTF MRNA IN NP TISSUES AND NPC CELL LINES

We detected *LTF* mRNA in seven NPC cell lines and eight NP tissues by semi-quantitative RT-PCR and real-time qPCR, respectively. The *LTF* mRNA level was normalized against the housekeeping gene *GAPDH*. The RT-PCR results showed *LTF* transcript was nearly undetectable in seven NPC cell lines (Fig. 1A), but it was stable in all the NP tissues (Fig. 1B). We further accurately evaluated the mRNA expression of *LTF* in the same samples by qPCR. The qPCR results revealed a remarkably decreased expression of *LTF* in seven NPC cell lines compared with NP tissues. Statistical analysis showed that *LTF* mRNA level in NPC cell lines detected by RT-PCR and qPCR was significantly lower than that in NP tissues ($P = 0.001$, $P = 0.001$, respectively. Data not shown).

GENETIC AND EPIGENETIC ALTERATIONS OF LTF GENE IN NPC CELL LINES

To elucidate the molecular mechanisms underlying the aberrant expression of *LTF* gene in NPC cells, we detected its genetic and epigenetic alterations in NPC cell lines, including LOH, mutation and promoter methylation status.

LOH analysis. Previous reports have suggested that allelic loss of chromosome 3p, including 3p21.3, is an early and critical molecular event in the tumorigenesis of NPC [Chan et al., 2000]. To determine whether the aberrant expression of *LTF* gene in NPC is due to genomic deletion, the allelic status of *LTF* was investigated by microsatellite analysis. As shown in Figure 2A, only one allelic loss was found at the D3S1478 site in CNE1 cell line and no allelic loss was found at other sites or other cell lines (Table I), that is, the total allele loss frequency of *LTF* gene in NPC cell lines was 14% (1/7).

Mutation analysis. By use of PCR-SSCP and subsequent sequencing analysis, we examined whether there existed mutations in the promoter region (−476 to −275 bp and −305 to −50 bp), exon 1 and

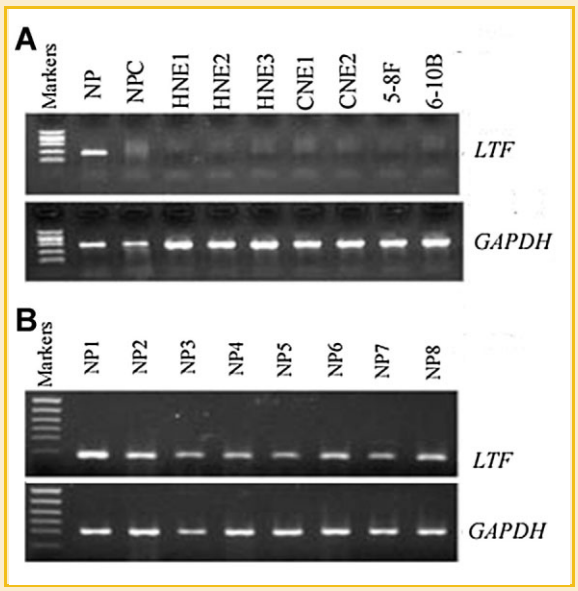

Fig. 1. Semi-quantitative RT-PCR analysis for detecting the mRNA levels of *LTF* in NPC cell lines (A) and NP tissues (B). Compared with NP tissues, NPC cell lines showed deficient expression of *LTF*.

exon 2 of *LTF* in 7 NPC cell lines. In consequence, only one mutation (1076delT) in the promoter region (−305 to −50 bp) was found in HNE1 cell line (Fig. 2B) and no mutation was found at other sites or in other NPC cell lines (Table I), resulting in a total mutation frequency of 14% in NPC cell lines (1/7).

Methylation analysis. It has been demonstrated that there is an association between methylation at the CpG sites of *LTF* gene promoter and its expression level [Teng et al., 2004]. To explore the potential role of CpG island methylation in the transcriptional silencing of *LTF* in NPC cells, we checked the methylation status of 13 CpG sites in a 168-bp promoter region of *LTF* in the NPC cell lines by MSP, which can specifically amplify the methylated and unmethylated alleles after the isolated DNA is chemically modified with sodium bisulfite. The MSP results showed that the *LTF* CpG island was methylated in 100% (7/7) of the NPC cell lines (Fig. 3A and Table I). As illustrated in Figure 3A, the unmethylated MSP product could also be amplified in CNE2 and 5-8F cell lines, indicating that *LTF* promoter was incompletely methylated in certain NPC cell lines. The MSP products were further sequenced. The sequencing results showed that all the cytosine residues were converted to thymines except for those in CpG dinucleotides, indicating the presence of methylated cytosines in these CpG dinucleotides (Fig. 3B). To explore the effect of promoter DNA methylation on expression of *LTF*, NPC cell lines were treated respectively with 5-aza-dC, a DNA demethylating agent. MSP and RT-PCR were used to determine the promoter methylation status and expression of *LTF*. The results showed that 5-aza-dC could effectively suppress methylation of *LTF* promoter as indicated by appearance of clear U-band in MSP experiment. DNA promoter hypermethylation was associated with the silencing of *LTF* gene in NPC cell lines, for DNA demethylation could reactivate or up-regulate expression of *LTF* gene (Fig. 3A).

Furthermore, we performed regional methylation analysis to directly analyze the role of promoter methylation in regulating *LTF* gene expression. Briefly, we constructed a series of luciferase reporter recombinants under the control of methylated (i.e., pGL3B/P1-116^{m}, pGL3B/P1-400^{m}, pGL3B/P1-615^{m}, pGL3B/P1-940^{m}, and pGL3B/P2-328^{m}) or unmethylated (i.e., pGL3B/P1-116^{c}, pGL3B/P1-400^{c}, pGL3B/P1-615^{c}, pGL3B/P1-940^{c}, and pGL3B/P2-328^{c}) promoter of *LTF*. Subsequently, the recombinants were transiently transfected into CNE2 cell lines, and the luciferase activities were measured. Consequently, pGL3B/P1-116 recombinant mapping −99 to +16 of *LTF* promoter exhibited relatively higher luciferase activity in CNE2 cells, suggesting that −99 to +16 region may be the core sequence of *LTF* promoter (data not shown). Once methylated, luciferase activities dramatically decreased. The ratios (mean ± SD) between luciferase activities of methylated and unmethylated promoter constructs were 0.086 ± 0.050 (pGL3B/P1-116^{m}), 0.017 ± 0.004 (pGL3B/P1-400^{m}), 0.037 ± 0.019 (pGL3B/P1-615^{m}), 0.743 ± 0.131 (pGL3B/P1-940^{m}), 0.838 ± 0.116 (pGL3B/P2-328^{m}), demonstrating that promoter methylation could veritably affect the transcription of *LTF* in NPC cells and this may be an important reason leading to aberrant *LTF* expression in NPC (Fig. 3C).

ESTABLISHMENT OF NPC CELL LINE STABLY EXPRESSING LTF

In order to explore the function of *LTF* gene in NPC, we re-expressed *LTF* in CNE2 cells by gene transfection method and then observed the biological changes of the *LTF*-transfected CNE2 cells (named CNE2-LTF). It was demonstrated that *LTF* gene was stably expressed at mRNA and protein levels in the CNE2-LTF cells as detected by RT-PCR and Western blotting (Fig. 4A,B). There exhibited two LTF bands in Figure 4B, which may be a consequence of different glycosylation patterns of LTF protein. Meanwhile, the CNE2-pc3.1 cells (named as CNE2-pc3.1) were also obtained by transfecting pcDNA3.1(−) blank vector into CNE2 cells. Immunocytochemical detection showed that LTF protein was present in the cytoplasm of CNE2-LTF cells (Fig. 4C), which is consistent with the earlier finding [Teng and Gladwell, 2006].

EFFECT OF LTF EXPRESSION ON BIOLOGICAL CHARACTERISTICS OF CNE2 CELLS

Numerous studies have demonstrated the negative effect of LTF protein supplementation on the growth of various cancer cells [Damiens et al., 1999; Xiao et al., 2004; McKeown et al., 2006; Shaheduzzaman et al., 2007]. Here we also evaluated the effect of *LTF* on NPC cell growth. First, the proliferative abilities of CNE2-LTF cells and CNE2-pc3.1 cells were measured by MTT, respectively. As the growth curve shown in Figure 5A, there were significant differences in the proliferative ability between these two groups of cells from the third day to the seventh day ($P < 0.05$), illustrating that *LTF* expression could lower the proliferation rate of CNE2 cells.

Meanwhile, the cell cycle profile of CNE2-LTF cells was analyzed by FCM. The results showed that *LTF* expression could block the cell cycle progression of CNE2 cells in G_1 phase (64.80 ± 1.00 vs. 55.13 ± 0.67), while the percentage of CNE2-LTF cells in S phase (27.43 ± 1.43 vs. 29.75 ± 1.31) and G_2-M phase (7.76 ± 1.01 vs. 15.13 ± 1.24) decreased as compared with CNE2-pc3.1 cells (Fig. 5B). Subsequently, the colony formation assay was carried

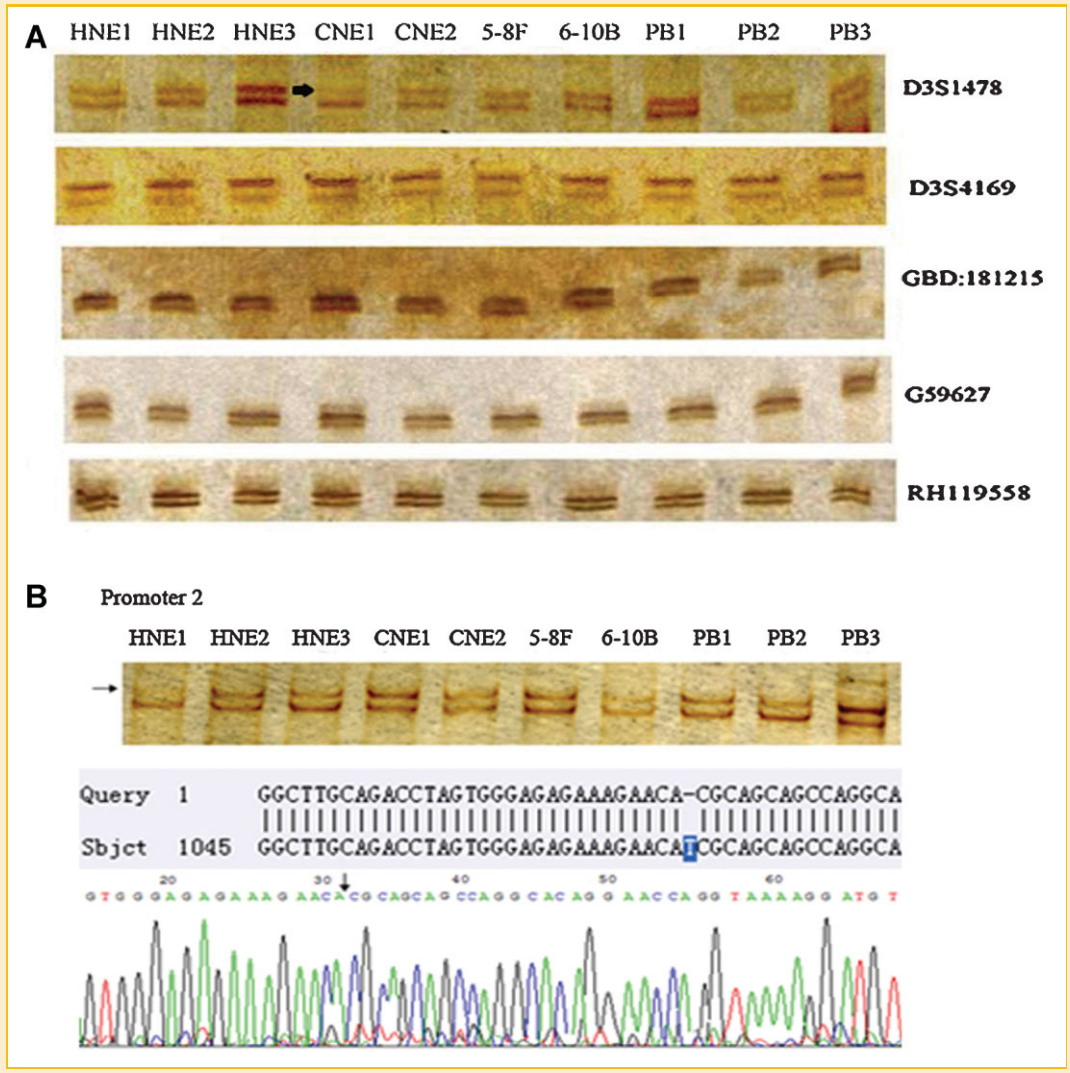

Fig. 2. LOH and mutation analysis results of *LTF*. The arrow in panel A indicates allelic loss of *LTF* in CNE1 cell line and the upper arrow in panel B indicates mutation of *LTF* in HNE1 cell line, compared with peripheral blood DNA of NPC patients (PB). The lower arrow in panel B indicates mutation point (1076delT) by DNA sequencing.

out to detect clonality of CNE2-LTF cells. Compared with CNE2-pc3.1 cells, CNE2-LTF cells exhibited a much lower cloning efficiency (28.0% vs. 54.7%, $P < 0.05$) (Fig. 5C), indicating that *LTF* had a negative effect on clonality of CNE2 cells.

TABLE I. Summary of *LTF* Alterations in NPC Cell Lines, Including Expression, LOH, Mutation, and Hypermethylation

	LTF gene			
NPC cell lines	Expression	LOH	Mutation	Hypermethylation
HNE1	−	−	+	+
HNE2	−	−	−	+
HNE3	−	−	−	+
CNE1	−	+	−	+
CNE2	−	−	−	+
5-8F	−	−	−	+
6-10B	−	−	−	+

TUMORIGENESIS ANALYSIS IN NUDE MICE

In order to compare the ability of tumor formation between CNE2-LTF cells and CNE2-pc3.1cells, we subcutaneously inoculated 5×10^6 cells of each kind to six nude mice (male and female was fifty-fifty) respectively. Tumor formation was examined after 6 weeks. All the nude mice were alive with the tumor developed. As shown in Figure 5D, CNE2-LTF cells manifested much weaker tumor formation potential compared with CNE2-pc3.1 cells ($P < 0.05$), *esp.* for the female mice. We examined the apoptosis state of tumor cells in transplanted nude mice by TUNEL method. The results showed that LTF could induce apoptosis to a considerable extent in nude mice inoculated with CNE2-LTF compared with that in control nude mice inoculated with CNE2-pc3.1. Meanwhile, the apoptosis rate of tumor cells in CNE2-LTF transplanted female mice was obviously higher than that in CNE2-LTF transplanted male mice. Immunohistochemistry detection demonstrated that the LTF expression level in female mice inoculated with CNE2-LTF was higher than that in male mice of the same group, which was consistent with the above

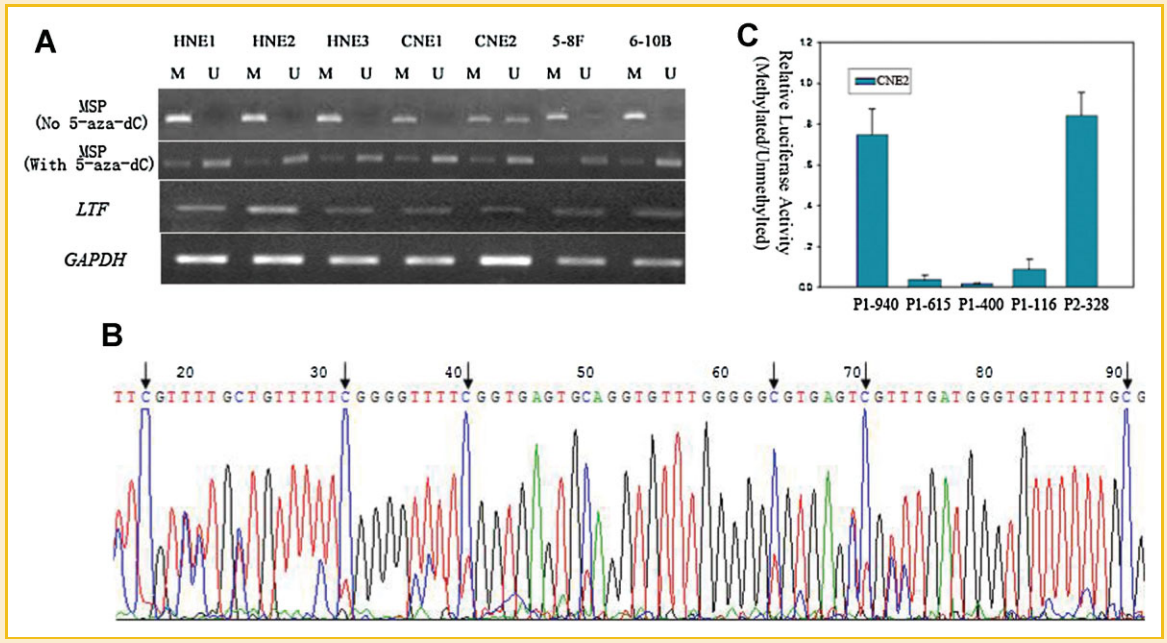

Fig. 3. Methylation, 5-aza-dC treatment and regional methylation results of LTF in NPC cell lines. A: MSP analysis and RT-PCR of LTF in NPC cell lines. M: MSP products using methylation-specific primers; U: MSP products using unmethylation-specific primers. The first and second panels shows MSP results of untreated and 5-aza-dC treated NPC cell lines, respectively. The third and forth panels shows RT-PCR results of LFT and GAPDH (as internal control) in 5-aza-dC treated NPC cell lines. B: Sequencing analysis of methylated MSP product. Arrows show no C/T substitutions at the CpG sites because of methylation protection. C: Methylation of LTF promoter inhibited its activity in CNE2 cells. The data show the ratios between luciferase activities of methylated and unmethylated promoter constructs (mean ± SD).

results and the fact that tumors in female mice were smaller than those in male mice in both CNE2-LTF group and CNE2-pc3.1 group (Fig. 6).

DISCUSSION

Numerous cytogenetic and allelotype studies have shown that losses of 3p occur frequently in NPC and other cancers. Functional evidence provided by microcell-mediated monochromosome transfer has precisely indicated that the 3p21.3 region displays strong tumor suppressor activity in NPC cell line [Cheng et al., 1998]. To date, several candidate genes mapped on chromosome 3p21.3 region have been studied [Liu et al., 2003; Chow et al., 2004; Qiu et al., 2004; Zhou et al., 2005, 2009], but no credible NPC candidate gene has been identified yet.

The human *LTF* gene, which is located at 3p21.3, comprises 17 exons, encodes a 2.4-kb mRNA and has been regarded as a candidate TSG in lung cancer [Finkbeiner et al., 1993; Iijima et al., 2006] and NPC [Yi et al., 2006], etc. In this study, we utilized NPC cell lines as the subjects in our experiments to evaluate the role of *LTF* gene in NPC. First, semi-quantitative RT-PCR and qPCR analyses of *LTF* in NPC cell lines showed remarkable *LTF* down-regulation, compared with NP control tissues, which is consistent with the results in NPC tissues [Yi et al., 2006; Zhou et al., 2008]. Secondly, to find the reasons leading to deficient expression of *LTF* in NPC cell lines, we carried out LOH and gene mutation studies to investigate the genetic changes of *LTF*. Our results showed that only one allelic loss was found at one site (D3S1478) in one NPC cell line (CNE1) and only one mutation site (1076delT) was found in another NPC cell line (HNE1), both the frequency of LOH and gene mutation of *LTF* in NPC cell lines were 14% (1/7). The results suggested that genomic deletion and gene mutation may not play important roles in *LTF* downregulation and epigenetic mechanism remains to be investigated.

More and more investigations have testified that promoter methylation is an alternative important way to attenuate gene expression. Published reports have indicated that hypermethylation of the promoter region is associated with *LTF* down-regulation in various human malignancies, including lung cancer, breast cancer, prostate cancer and leukemia, etc. We have previously found that promoter hypermethylation of *LTF* exists in 63.6% of primary NPC tissues but not in NP tissues, providing evidence that promoter hypermethylation should play a main role in regulating *LTF* expression in NPC. In NPC cell lines, we also found that promoter hypermethylation existed in each NPC cell line (100%, 7/7) through MSP and sequencing analyses. In addition, we also inhibited *LTF* promoter methylation level by 5-aza-dC (a DNA demethylating agent) to detect the effect of methylation on *LTF* expression in these cell lines. After 5-aza-dC treatment, we found the methylation level of *LTF* gene decreased remarkably and *LTF* exhibited re-expression in all cell lines, further supporting the idea that promoter methylation should be the key mechanism causing *LTF* down-regulation in NPC cell lines. Regional methylation analysis of *LTF* promoter with luciferase reporter constructs indicated that methylation of *LTF* promoter indeed significantly decreased its

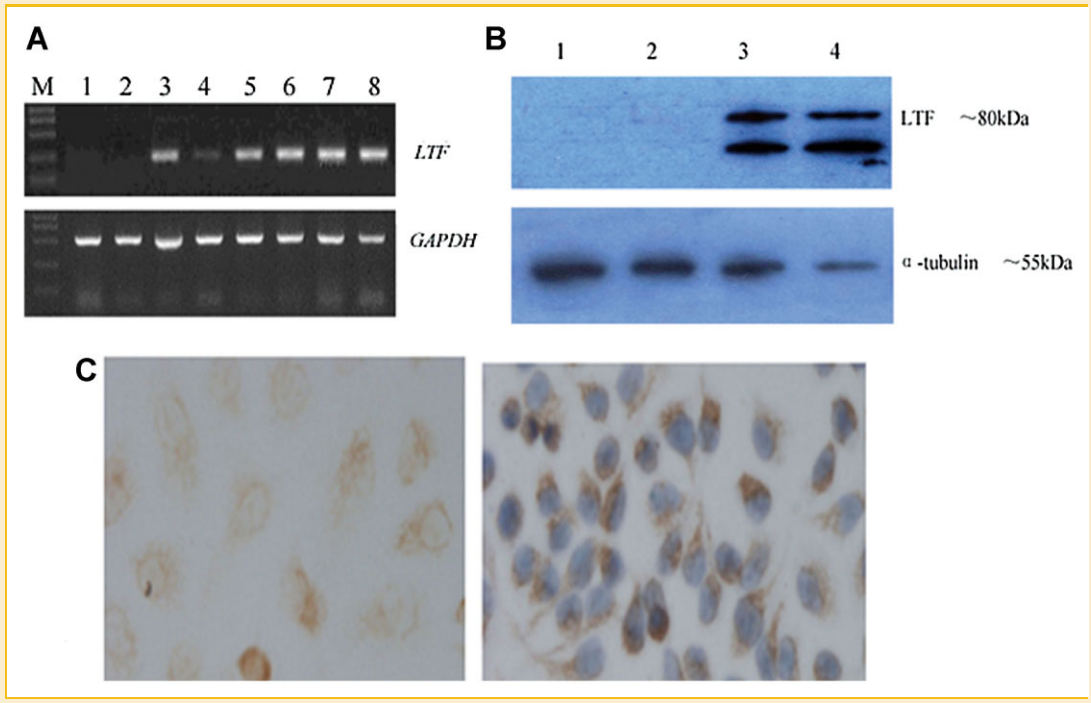

Fig. 4. Expression of *LTF* at the mRNA (A) and protein (B,C) levels in the CNE2-LTF cells. A: M: PCR marker, 1, 2: undetectable *LTF* mRNA expression in CNE2 cells and CNE2-pc3.1 cells; 3–8: *LTF* mRNA expression in six different CNE2-LTF cell clones. B: 1,2: Undetectable LTF protein expression in CNE2 and CNE2-pc3.1 cells; 3, 4: strong LTF protein expression in two different CNE2-LTF cell clones. C: Immuno-staining showed that LTF protein was present in the cytoplasm of CNE2-LTF cells. Left image: uncounterstained with hematoxylin; Right image: counterstained with hematoxylin (DAB staining, 400×).

activity. We concluded that promoter hypermethylation may be a crucial mechanism causing inactivation of *LTF* in NPC cell lines compared with LOH and gene mutation, but more aspects remain to be elucidated concerning the mechanism(s) leading to *LTF* inactivation, for example, histone deacetylation, regulation of transcription factors [Iijima et al., 2006]. Unexpectedly, RT-PCR results showed *LTF* transcript was nearly undetectable in CNE2 and 5-8F, while the MSP analysis of *LTF* showed that the *LTF* promoter was incompletely methylated in these two cell lines. It is possible that there are other mechanism(s) leading to *LTF* inactivation in these NPC cell lines, whereas more work is needed to be done for clarifying this. As for CNE1, there was an allelic loss at D3S1478 site except promoter methylation. We think both methylation and LOH may be responsible for the inactivation of *LTF* gene in this cell line. Upon 5-aza-dC treatment, *LTF* could partially restore its expression, suggesting promoter methylation still played an unnegligible role in *LTF* inactivation in CNE1 cell line.

The absence of *LTF* expression in a high percentage of NPC cell lines and NPC tissues prompted us to examine the consequences of forced *LTF* expression on the phenotype of NPC cells. As previous studies about negative effects of LTF protein in cancer cell have focused on the methods of supplementation in culture media, here we showed that restoration of *LTF* mRNA and protein expression in NPC cells by gene transfection resulted in significant inhibition of cell growth and reduced colony-forming capacity compared with non-transfected parental cells. In addition to CNE2 cells, 5-8F cells were also transfected with *LTF* gene and similar results of FCM analysis, MTT assay and colony formation assay were obtained (data not shown), in support of a general role of *LTF* in NPC.

We evaluated the alterations in cell cycle profile of NPC cells in response to *LTF* transfection to assess the underlying mechanisms of LTF protein-mediated anti-tumorigenic activity. *LTF* transfected NPC cells exhibited an elevated G1 fraction with fewer cells in S phase and G2-M phase, suggesting a role of specific cell cycle regulatory mechanism in LTF-mediated cell growth inhibition. Similar observations were reported in breast carcinoma cells [Damiens et al., 1999] and head and neck cancer cells [Xiao et al., 2004]. Some reports have suggested that LTF protein can regulate the levels of cyclin, cyclin-dependent kinase, cyclin-dependent kinase inhibitors, tumor suppressors (p53 and Rb) to affect the progression of cell cycle [Oh et al., 2004; Xiao et al., 2004; Son et al., 2006], but Zhou et al. think LTF treatment can modulate the mitogen-activated protein kinase (MAPK) pathway in NPC cells without affecting p53 and STAT3 expression. Usually, LTF protein is a secretary protein and exists in biological fluids; however LTF protein has also been discovered to function as a transcription factor [He and Furmanski, 1995]. Our result of immunocytochemistry showed that the expressed recombinant human LTF protein was predominantly present in the cytoplasm, so we speculated that the recombinant human LTF protein could enter the cells by some ways [e.g., autocrine Oh et al., 2001, 2004] and be transported into the nucleus to regulate gene expression. But more work is required to be done to confirm it.

In vivo experiments showed that the tumor formation ability of CNE2-LTF cells was significantly lower than that of the control cells,

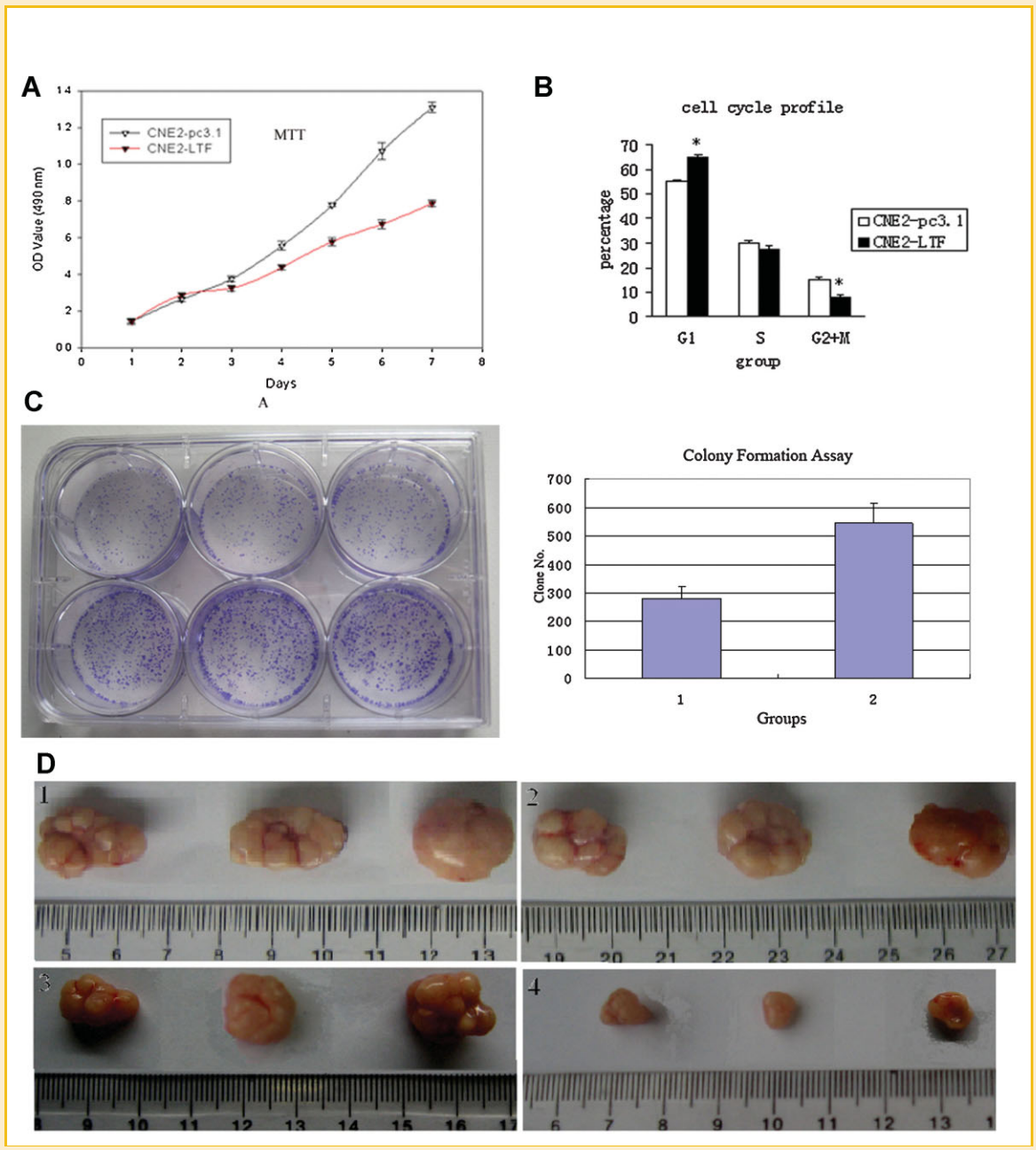

Fig. 5. Effect of LTF expression on biological characteristics of CNE2 cells. A: Effect of *LTF* expression on proliferative ability of CNE2 cells. The cells were cultured for the indicated days and cell proliferation was then assessed by MTT assay. The average absorbance values obtained from wells without cells served as a blank and were subtracted from all other results. The results revealed that CNE2-LTF cells proliferated much more slowly compared with CNE2-pc3.1 cells ($P < 0.05$). B: Flow cytometric analysis results showed that *LTF* expression could block the cell cycle progression of CNE2 cells in G1 phase, while the percentage of CNE2-LTF cells in G2-M phase decreased compared with CNE2-pc3.1 cells. $^{*}P < 0.05$ (CNE2-LTF cell group vs. CNE2-pc3.1 cell group). C: Colony formation assay of CNE2-LTF cells (1) and CNE2-pc3.1 cells (2). The results represent the mean $\pm$ SD values of colony number from three independent experiments (Colony number: 1: 280 $\pm$ 43; 2: 547 $\pm$ 69). D: Tumor formation assay of CNE2-LTF cells and CNE2-pc3.1 cells in nude mice. The picture showed the excised tumors from male nude mice with CNE2-pc3.1 (1), male nude mice with CNE2-LTF (2), female nude mice with CNE2-pc3.1 (3) and female nude mice with CNE2-LTF (4), respectively. Statistic analysis indicated that the size and weight of the tumors in nude mice inoculated with CNE2-LTF were significantly smaller and lighter than those in control mice ($P < 0.05$), *esp.* for the female mice.

indicating *LTF* may be an important candidate tumor suppressor. However, we did not observe occurrence of tumor metastasis in any nude mouse, which may be due to the main shortage of subcutaneous transplanted model, as previous literature reported that tumor cells rarely metastasized to distance sites when they were inoculated subcutaneously [Kubota, 1994]. It is interesting that the tumors in female mice were apparently smaller than those in male mice no matter in experimental groups or in control groups and the female experimental group developed tumors with minimum size. We speculate that estrogen in female mice may exert additional

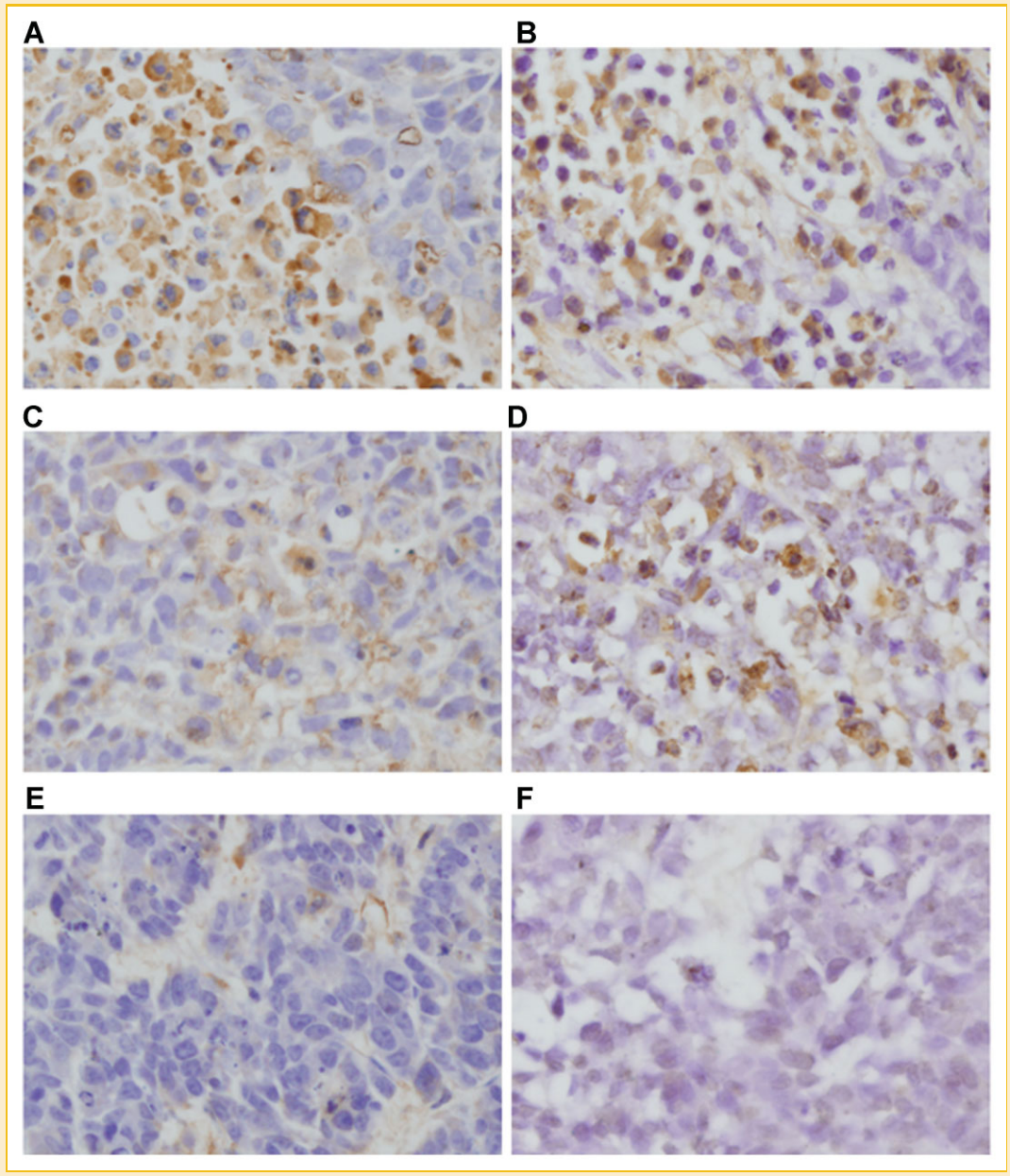

Fig. 6. Immunohistochemistry detection of LTF expression (A,C,E) and TUNEL analysis (B,D,F) of tumor cells in nude mice. A,B: Tumor cells from female nude mice inoculated with CNE2-LTF. C,D: Tumor cells from male nude mice inoculated with CNE2-LTF. E,F: Tumor cells from nude mice inoculated with CNE2-pc3.1.

inhibitory effect on the xeno-transplanted tumors through upregulating the expression of tumor suppressor genes including *LTF* [Teng, 1999; Zhang and Teng, 2000; Stokes et al., 2004] and/or downregulating the expression of some certain oncogenes. For confirming this, we further detected the LTF expression in tumor xenografts from male and female nude mice by immunohistochemistry. As a result, we found that the LTF expression level in transplanted female mice was really higher than that in male mice, in favor of our speculation that estrogen could upregulate LTF expression and higher LTF expression exerted additional inhibitory effect on the xeno-transplanted tumors in female mice. As some articles have reported that LTF protein can induce apoptosis in some kinds of cancer cells [Fujita et al., 2004; Artym, 2006], our TUNEL results indicated that the apoptosis rate of tumor cells in nude mice inoculated with CNE2-LTF was obviously higher than that of control tumor cells, which can largely explain the inhibitory effect of LTF on tumorigenesis. A recent report has shown that 700 μg/ml of human LTF can significantly reduce the cell viability, increase the caspase 3 and 8 activities and decrease the protein expression of phosphorylated extracellular-signal-regulated kinase 1/2 (ERK1/2) and Bcl-2 in PC12 neuronal cells [Lin et al., 2005]. A similar mechanism may underlie the apoptosis-inducing activity of LTF on tumor cells. We

observed that the effect of LTF on apoptosis of tumor cells in female mice was more intense than that in male mice, in coincidence with the fact that the tumors in inoculated male mice were manifestly larger than those in female mice. We inferred that higher LTF expression level may also contribute to the higher apoptosis rate and smaller tumor size in female nude mice. As indicated by in vitro cell cycle analysis, we also suppose that inhibitory effect on tumor cell proliferation of LTF may be another possible reason causing retarded tumorigenesis in LTF transfection group.

Taken together, this study indicates that *LTF* may participate in NPC carcinogenesis as a tumor suppressor gene, for it is frequently downregulated in NPC cells and exhibits obvious anti-tumorigenic activity when it is re-expressed.

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