Supplemental data

LIF regulates p70S6K to promote progressiveness and radioresistance of NPC

Table of Content:

Figure S1

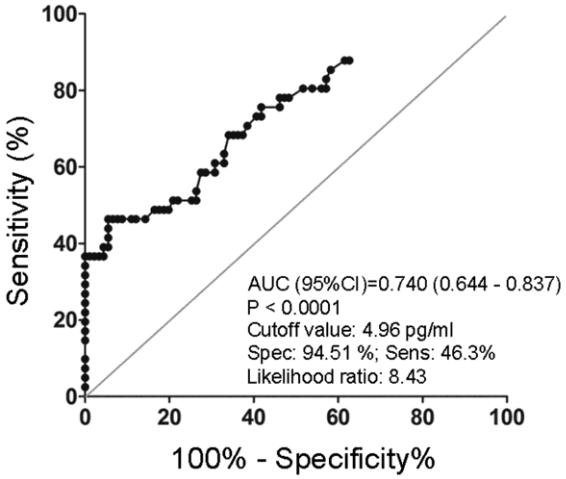

Figure S1. Receiver operating characteristic (ROC) curve analysis. The ROC curve was used to determine the serum LIF cutoff value that could be used to distinguish patients with complete tumor remission from those that developed local recurrence after radiotherapy.

Figure S2

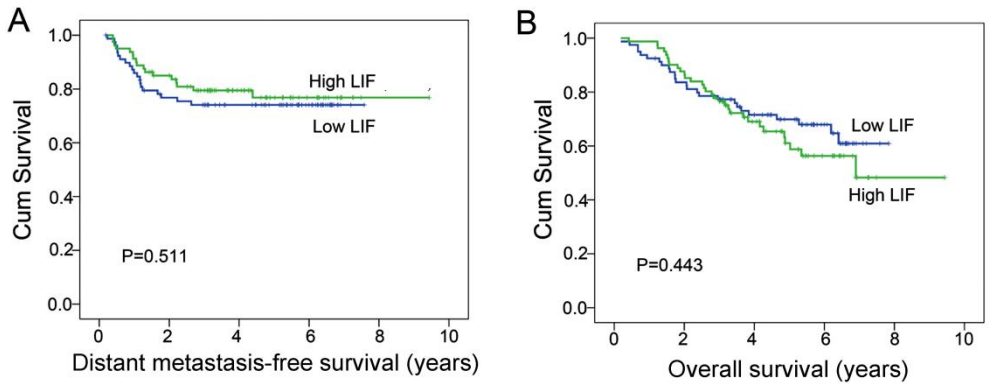

Figure S2. Kaplan–Meier survival curves of NPC patients based on serum LIF levels.

(A) Distant metastasis-free survival. (B) Overall survival.

Figure S3

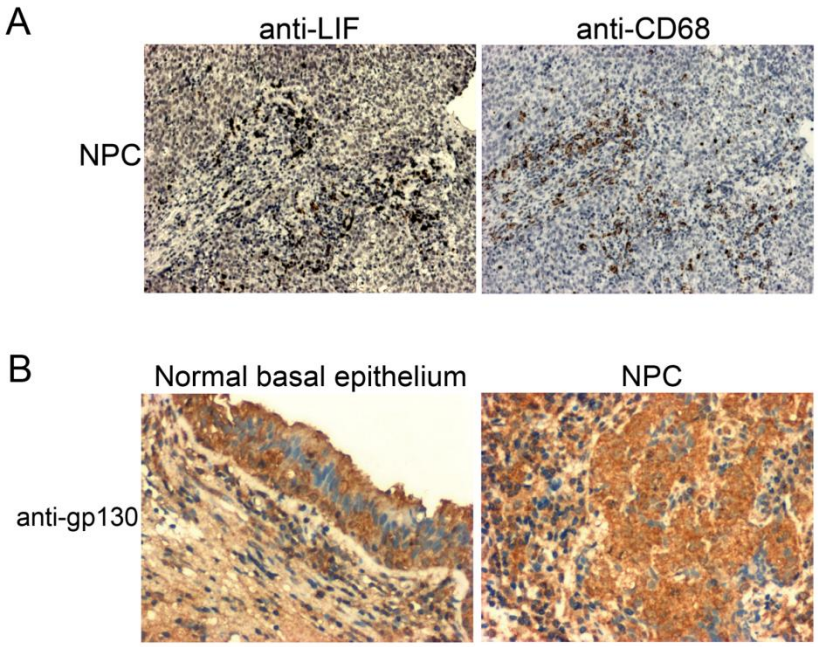

Figure S3. IHC analysis of NPC biopsy samples. (A) Expression of LIF and CD68 (a human macrophage marker). IHC images revealed strong LIF immunoreactivity in infiltrated macrophages. (B) Expression of gp130 in NPC. Representative images of gp130 protein expression in NPC tumor cells and the adjacent basal layer. Paraffin-embedded sections were stained with antibodies against LIF, CD68, or gp130 (orange to brown) and counterstained with hematoxylin (blue). Magnification × 200.

Figure S4

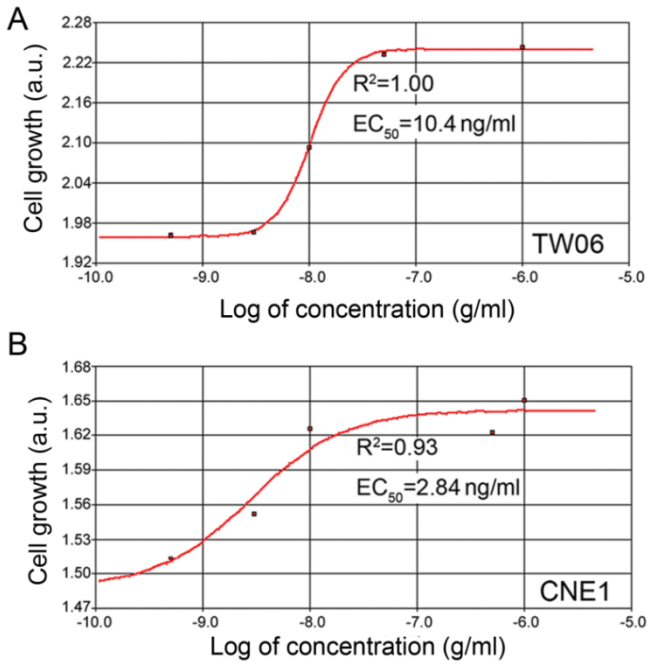

Figure S4. Determining the EC_{50} values of LIF for two NPC cell lines: (A) CNE1 cells and (B) TW06 cells. EC_{50}values were calculated from the dose-response curves (0.5 ng/ml, 3 ng/ml, 10 ng/ml, 50 ng/ml, and 1μg/ml) using the RTCA software. Cell growth values are displayed with respect to the logarithm of concentration.

Figure S5

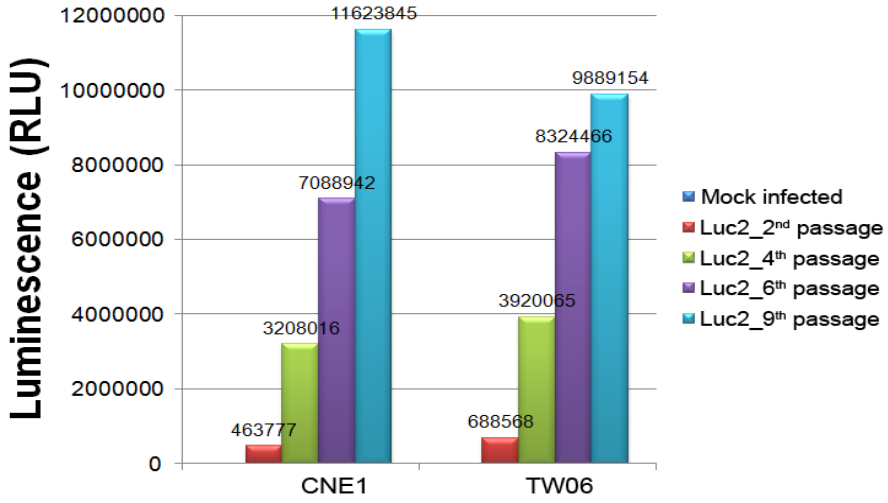

Figure S5. *In vitro* bioluminescence assay. Selected CNE1_Luc2 and TW06_Luc2 clones were analyzed for their luciferase activity at several culture passages as indicated. The 9th culture passages of isolated clones were used for *in vivo* studies.

Figure S6

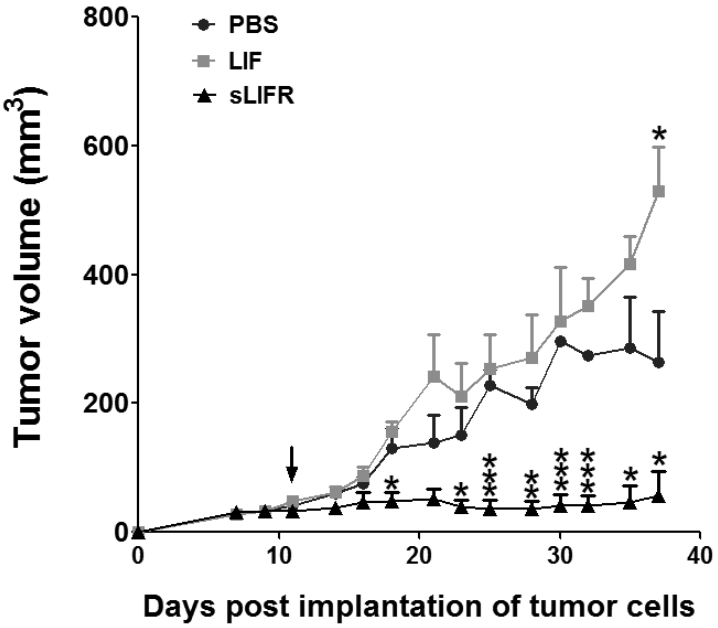

Figure S6. LIF promotes the tumor growth in CNE1 xenografts. Drug administration was started on day 11 post implantation (LIF: 150-200 ng/20 μl PBS, twice/week × 4 weeks; sLIFR: 1-2 μg/20 μl PBS, twice/week × 4 weeks; PBS: 20 μl, twice/week × 4 weeks). Values are presented as means and SEM (n = 5 mice per group; $^{*}P< 0.05$, $^{**}P< 0.01$, $^{***}P< 0.001$, paired t-test, compared with PBS-treated group).

Figure S7

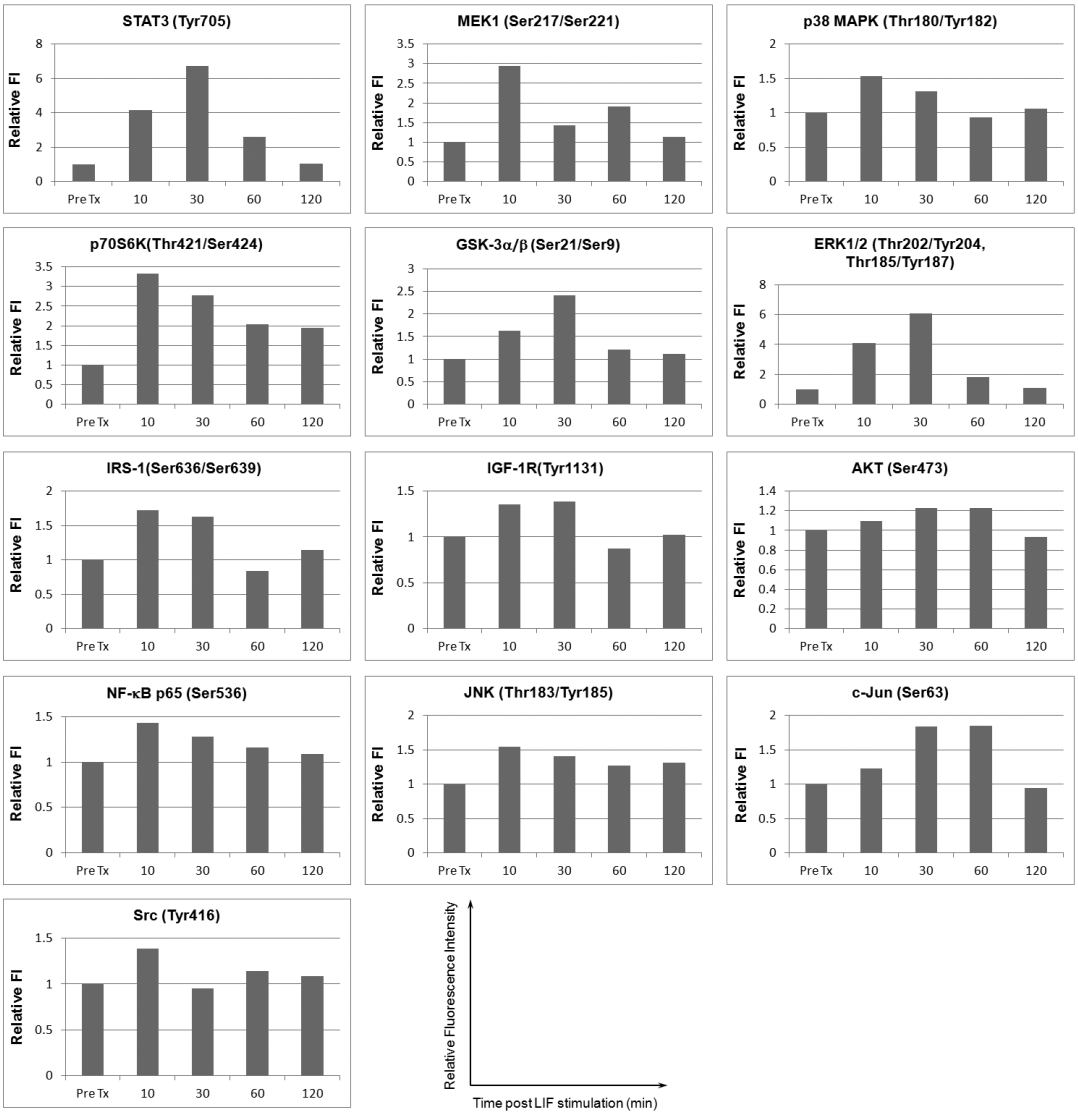

Figure S7. LIF activates multiple signaling proteins in CNE1 cells. Phosphorylation levels were measured using multiplex phosphoprotein assays (BioRad). Protein lysates were harvested at the indicated time points.

Figure S8

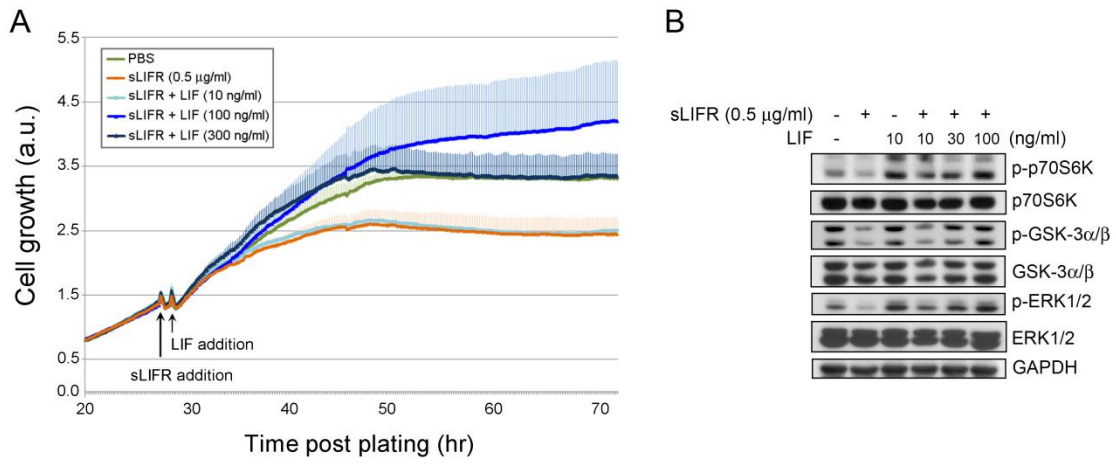

Figure S8. Administration with increased doses of LIF overcomes sLIFR-mediated effects in NPC cells. (A) Real-time cell proliferation assays of TW06 cells treated with LIF, sLIFR or PBS (control). Black arrows indicate the time at which LIF or sLIFR were added. Values are presented as means and SD of triplicate experiments. (B) Western blotting analyses of p70S6K signaling molecules in TW06 cells. sLIFR (0.5 μg/ml) was added to the growth medium 2 hours prior to LIF treatment. Protein lysates were harvested 10 minutes after LIF treatment. GAPDH was used as a loading control.

Figure S9

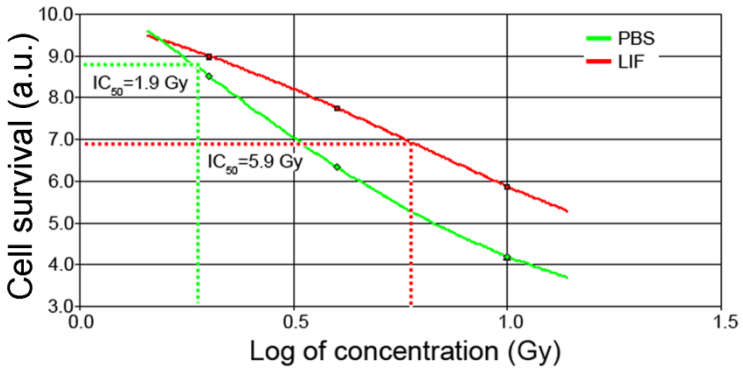

Figure S9. LIF treatment increases the survival of TW06 cells subjected to ionizing radiation. IC_{50} of TW06 cells after γ-ray irradiation was calculated. The IC_{50}values for PBS or LIF (10 ng/ml)-treated cells were calculated at 52 hours after exposure to 2, 4, and 10 Gy γ-ray using the RTCA software. Each experiment was performed in triplicate.

Figure S10

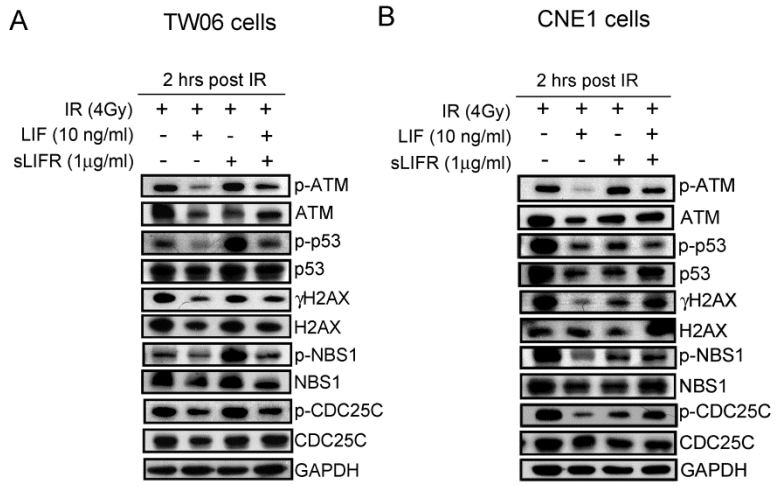

Figure S10. LIF modulates the phosphorylation levels of DNA damage-responsive molecules in TW06 cells (A) and CNE1 cells (B) subjected to 4-Gy irradiation. Protein lysates were harvested at 2 hours after irradiation. GAPDH was used as the loading control**.**

Figure S11

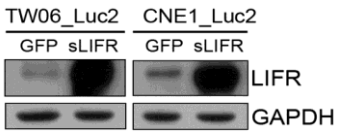

Figure S11. Western blotting of LIFR in NPC cells infected with lentivirus expressing the extracellular domain of human LIFR (Gln45-Ser833) or GFP (control). GAPDH was used as the loading control. The selected CNE1_Luc2_sLIFR cells and CNE1_Luc2_GFP cells were used for *in vivo* studies.

Figure S12

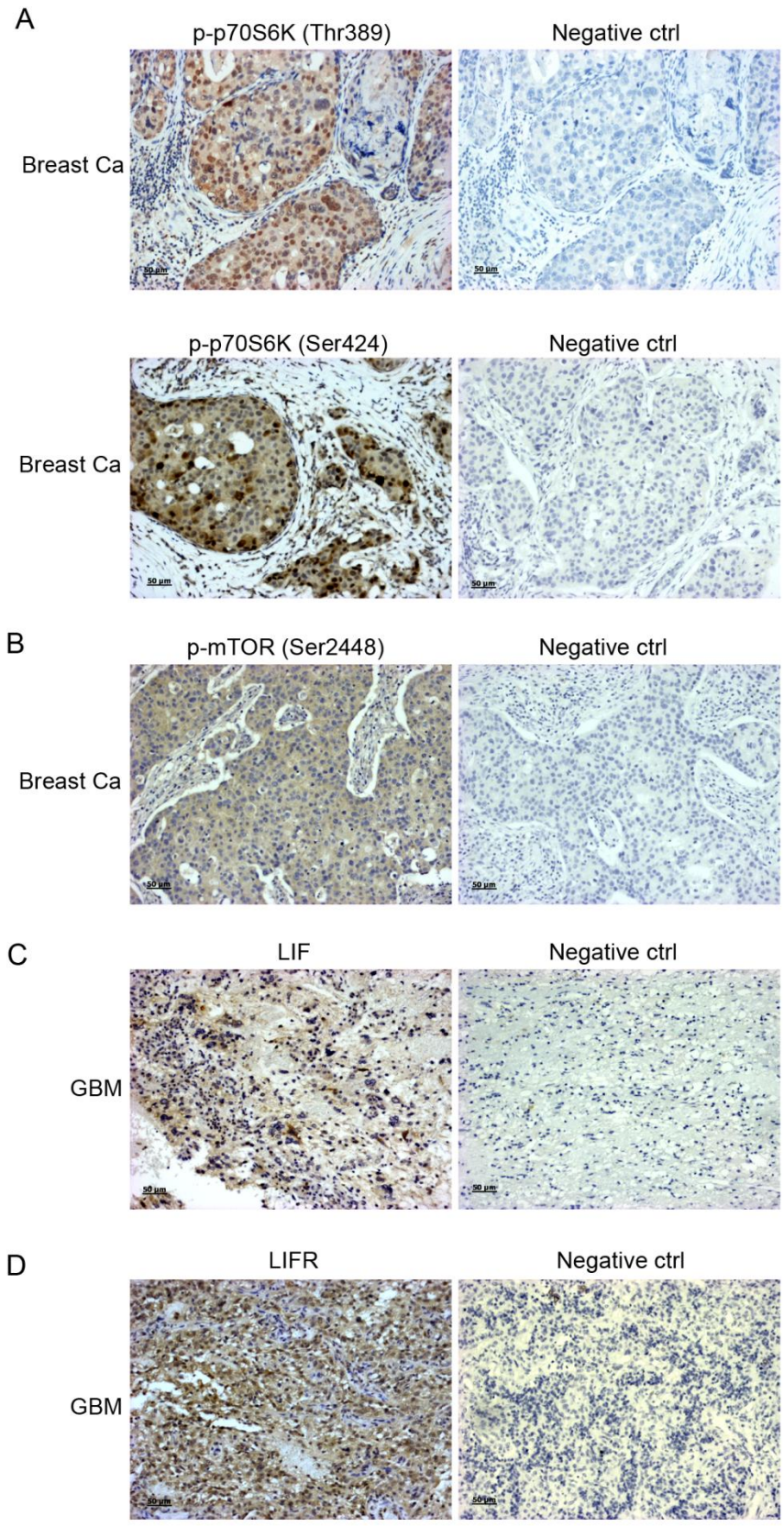

Figure S12. Control staining of immunohistochemical analysis. (A-B)

Paraffin-embedded breast cancer sections were used as positive control tissue of phospho-p70S6K (Thr389 and Ser424) (A) and phospho-mTOR (Ser2448) (B). (C-D) Immunohistochemical detection of LIF (C) and LIFR (D) expression in glioblastoma multiforme (GBM) tissue sections. The primary antibody was omitted from negative controls.

Table S1. Analysis of 20 cytokines in serum of NPC patients compared with normal donors (Mann-Whitney test).

Cytokines	Normal (n=28) vs.		
	Recurrence (n=22)	**Metastasis (n=23)**	**All NPC (n=71)**
LIF	< 0.0001	0.0994	0.0172
CXCL9 (MIG)	< 0.0001	0.0033	0.0006
IL-10	0.0043	0.2505	0.2174
IL-6	0.0072	0.1715	0.1406
SCF	0.0342	0.0863	0.0401
IL-12	0.1374	0.5198	0.6442
IL-1β	0.1379	0.3143	0.4845
IL-16	0.1807	0.3586	0.623
IL-8	0.2064	0.7833	0.7507
IL-18	0.3906	0.0922	0.1174
CTACK	0.4757	0.8828	0.7125
IL-3	0.6142	0.5249	0.8958
SCGF-β	0.6321	0.698	0.2354
MCP-3	0.6548	0.58	0.4262
HGF	0.8374	0.5637	0.7469
MIP-1α	0.8417	0.2279	0.0721
M-CSF	0.8417	0.4499	0.2404
GRO-α	0.8641	0.3109	0.1954
TNF-β	0.9454	0.4429	0.2292
IL-1α	0.9844	0.5404	0.2704

Table S2. Correlations between LIF serum levels and clinicopathological factors in NPC patients.

Characteristic	Patient number (n = 161)	LIF (pg/ml) (Median ± SD)	P-value[B]
Gender			
Male	113	2.80 ± 1.02	0.315
Female	48	2.02 ± 0.90	
Age[A]			
＜ median	81	1.94 ± 0.51	0.086
≧ median	80	3.44 ± 1.60	
Overall stage (2002 AJCC criteria)			
I - II	41	2.31 ± 0.49	0.57
III - IV	120	2.90 ± 1.66	
T-stage (2002 AJCC[C] criteria)			
T1 - T2	74	2.09 ± 0.68	0.275
T3 - T4	87	3.17 ± 1.61	
N-stage (2002 AJCC criteria)			
N0 - N1	76	2.60 ± 1.05	0.852
N2 - N3	85	2.84 ± 1.02	

[A] Median age is 46.77 years. [B]P values were calculated using the Independent Samples t-test. [C]AJCC = American Joint Committee on Cancer.

Table S3. Primers information

Symbol	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	product size (bp)	Annealing temperature, time (cycle)
LIF	CACTCACTTTGCACCTTTC	GCCACATCCTCGTCTTG	155	60, 7 sec (40)
LMP1	ACAAAACTGGTGGACTC	GTCTGCCCTCGTTGGA	188	63, 8 sec (45)
COL4A6	AGAGGTCAGCACACAT	GCTTTACTTTGAACCAGGC	330	64, 14 sec (40)

Supplemental Methods

Detection of cytokine levels in serum samples

The serum levels of 20 human cytokines (CTACK, CXCL9, GRO-α, HGF, IL-10, IL-12, IL-16, IL-18, IL-1α, IL-1β, IL-3, IL-6, IL-8, LIF, MCP-3, M-CSF, MIP-1α, SCF, SCGF-β, and TNF-β) were determined using a magnetic bead-based cytokine assay (Bio-Rad) as previously described (1). Appropriate human cytokine standards were included as positive controls. For the discovery of NPC-specific serum biomarkers, we first compared the serum levels of cytokines in healthy individuals (n = 28) and NPC patients (n = 71). Following the identification of LIF as the most differentially regulated cytokine in NPC patients diagnosed with recurrence, we enrolled 90 additional NPC patients. In total, serum LIF levels were analyzed in 161 NPC patients. The lower limit of detection for this assay was 0.04 pg/ml.

Reagents

Rapamycin (30 nM), Ly294002 (30 μM) (both from Calbiochem), and everolimus (20 nM) (Sigma-Aldrich) were used to block specific signaling pathways. Human LIF and human soluble LIFR (sLIFR) were purchased from Sigma-Aldrich.

Cell culture

Four NPC-derived cell lines, CNE1 (2), TW01 (3), TW06 (4), and C666-1 (5), were used in this study. CNE1, TW01, and TW06 cells were cultured in DMEM supplemented with 10% FBS (Invitrogen). C666-1 cells were maintained in RPMI 1640 supplemented with 10% FBS.

Multiplex phosphoprotein assay

To analyze the expression of the signaling phosphoprotein**s,** we used bead-based xMAP technology (Bio-Rad) to simultaneously detect 13 phosphoproteins, including STAT3 (Tyr705), MEK1 (Ser217/Ser221), p38MAPK (Thr180/Tyr182), p70S6K (THr421/Ser424), GSK-3α/β (Ser21/Ser9), ERK1/2 (Thr202/Tyr204, Thr185/Tyr187), IRS-1 (Ser636/Ser639), IGF-1R (Tyr1131), AKT (Ser473), NF-κB p65 (Ser536), JNK (Thr183/Tyr185), c-Jun (Ser63), Src (Tyr416). CNE1 cells were seeded at a density of 3×10^5 cells/well in the 6-well culture plate and allowed to grown overnight prior to LIF treatment. Protein lysates were harvested at various time points post LIF (10 ng/ml) stimulation. Detection of phosphoprotein levels and data analysis was performed according to the manufacturer's instructions.

Transfection

For small interfering RNA trasnfection, CNE1 cells were seeded at a density of 3×10^5 cells/well in the 6-well culture plate for 16 hours prior to transfection. Cells

were transfected with four small interfering RNA (siRNA) against mTOR (target sequences: 5'-CCAAAGCACUACACUACAA-3', 5'-GCAGAAUUGUCAAGGGAUA-3', 5'-CAAAGGACUUCGCCCAUAA-3', 5'-GGCCAUAGCUAGCCUCAUA-3') or a non-targeting control siRNA (Dharmacon) using DharmaFECT-1 reagent (Dharmacon), as described by the manufacturer, to achieve a final siRNA concentration of 90 nM. For exogenous LMP1 expression, cells were transiently transfected with vector plasmids, wild-type LMP1 plasmids, or LMP1 mutant plasmids using Fugene HD transfection reagents (Roche). Cell-free culture supernatants, RNA, or protein lysates were harvested at 24 hours post transfection.

Western blotting

Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. Blots were probed with primary antibodies against phospho (Thr202/Tyr204)-p44/42 MAPK (ERK1/2) (1:4000), phospho (Ser21/9)-GSK-3α/β (1:3000), phospho (Thr421/Ser424)-p70S6K (1:1000), phospho (Thr389)-p70S6K (1:2000) (all from Cell Signaling Technology), phospho (Ser2448)-mTOR (1:3000), phospho (Tyr705)-STAT3 (1:3000), phospho

(Ser1981)-ATM (1:3000), phospho (Ser392)-p53 (1:500), phospho (Ser216)-CDC25C (1:2000), phospho (Ser139)-H2AX (1:1000), phospho (Ser343)-NBS1(1:2000), LIF (1:200), GSK-3α/β (1:2000), p70S6K (1:5000), STAT3 (1:3000), CDC25C (1:4000), p53 (1:2000), ATM (1:3000), H2AX (1:1000), NBS1(1:2000), phospho (Ser32)-IκB-a (1:2000) (all from Epitomics), LIFR (1:200), gp130 (1:200), p44/42 MAPK (ERK1/2)(1:1000) (Santa Cruz Biotechnology), or GAPDH (1:3000) (Abcam), followed by incubation with horseradish peroxidase-conjugated secondary antibody and development with enhanced chemiluminescence detection reagents.

5-ethynyl-2-deoxyuridine (EdU) incorporation assay

The effect of LIF or sLIFR on DNA synthesis of NPC cells was evaluated using Click-iT® EdU Alexa Fluor® 488 EdU incorporation kit (Invitrogen) according to manufacturer's protocol. NPC cells were treated with LIF or sLIFR for 24 hours followed by EdU (10 μM) labeling for 3 hours. At least one thousand nuclei were counted for each experimental group. The number of EdU positive (EdU^+) cells was determined using ImageJ software. The percent of EdU^+ cells was calculated by dividing the number of EdU^+ cells by the total number of nuclei stained with Hoechst 33342 DNA dye.

Apoptosis assay

IR-induced apoptosis was determined using CellEvent™ Caspase-3/7 Green Detection Reagent (Invitrogen). Live NPC cells (CNE1 and TW06 cells) were labeled with 3 μM caspase-3/7 detection reagent at 96 hours after 4 Gy γ-irradiation, followed by Hoechst 33342 staining for 15 minutes according to manufacturer's instructions. Cells were analyzed on an inverted fluorescence microscope (Zeiss Axiovert 200 M). At least twenty-five distinct fields were counted under the 10x objective for each experimental group. The percent of cells positive for activated caspase-3/7 was analyzed using ImageJ software.

Irradiation

CNE1 or TW06 cells were seeded and allowed to grow overnight at 37℃. Cells were then irradiated at a given dose as indicated in the text and/or the respective figure legends using a Gammacell 3000 irradiator (Best Theratronics, Ottawa, Canada) at a dose rate of approximately 5.0 Gy/min.

Generation of NPC cell lines expressing soluble LIFR

The lentiviral vectors expressing the extracellular domain of human LIFRα (Gln45-Ser833) or GFP (control) was conducted using MultiSite Gateway Three Fragment Vector Construction Kit (Invitrogen), following the manufacturer's instruction. Briefly, the coding sequences of the extracellular domain of human LIFRα (sLIFR) were amplified using the following primers: 5'attB1_sLIFR, GGGGACAAGTTTGTACAAAAAAGCAGGCTCATTGCACAGATGCAGAAAAA GGGGGC; 3'attB2_sLIFR, GGGGACCACTTTGTACAAGAAAGCTGGGTTTAAGAATTTTCCTTTGTCACC ACATAC. The PCR products were cloned into the intermediate vector pDONR221, and subsequently, together with pENTR5'/CMVp, introduced into the lentiviral expression vector pLenti6.4/R4R2/V5-DEST using Gateway recombination technology (Invitrogen). Replication-incompetent lentivirus was produced by cotransfection of the pLenti6.4_sLIFR or pLenti6.4_GFP expression vector and packaging plasmids pLP1, pLP2, and pVSV-G into 293FT cells using the Lipofectamine 2000 (Invitrogen). Viral supernatant was harvested 48 hours after transfection, filtered through a 0.45 μm cellulose acetate filter, and stored in aliquots at -80℃. CNE1_Luc2 cells or TW06_Luc2 cells were incubated with lentivirus supernatant for 7 hours in the presence of 6 μg/ml of polybrene (Sigma-Aldrich).

Transduced cells were selected by the addition of 5 μg/ml of blasticidin (Invitrogen) to the medium. The selected clones (CNE1_Luc2_sLIFR and TW06_Luc2_sLIFR) were maintained in complete medium supplemented with 3 μg/ml of puromycin and 3 μg/ml of blasticidin.

Plasmids

Plasmid constructs of the wild-type Flag-tagged LMP1,ΔYYD (which had the last three amino acid of LMP1 deleted), mCTAR1 (which the CTAR1 TRAF-binding motif 'PXQXT' had been mutated to 'AXAXT') and ΔCTerm (C-terminal deleted LMP1) were generated as previously described (6).

Quantitative real-time reverse transcription-polymerase chain reaction

RNA extraction and quantitative real-time reverse transcription-polymerase chain reaction (QRT-PCR) were performed as previously described (7). Primers were designed using LightCycler probe design software (Roche Applied Science) (Table S3). Expression was expressed relative to that of COL4A6, which showed unchanged expression levels across NPC microarray experiments (GSE14262) (7). All assays were repeated in duplicate.

References

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