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Interleukin 7/interleukin 7 receptor induce c-Fos/c-Jun-dependent vascular endothelial growth factor-D up-regulation: A mechanism of lymphangiogenesis in lung cancer

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ABSTRACT

Interleukin 7 (IL-7) is known to promote lymphangiogenesis. To study the relationship between IL-7 and the lymphangiogenic factor, vascular endothelial growth factor (VEGF)-D, in human lung cancer cells and its impact on the prognosis of lung cancer patients, we investigated how IL-7 regulates VEGF-D. We found that, in lung cancer cell lines, IL-7/IL-7 receptor (IL-7R) increase the expression of VEGF-D and phosphorylation of c-Fos/c-Jun, induce c-Fos and c-Jun heterodimer formation, and enhance c-Fos/c-Jun DNA binding activity to regulate VEGF-D. In addition, the expression levels of IL-7 and IL-7R correlated well with that of VEGF-D, lymphatic vessels density (LVD), clinical stages, lymph node metastasis, and poor prognosis in 100 human non-small cell lung cancer (NSCLC) specimens analysed. Taken together, our results provide evidence that IL-7/IL-7R induce VEGF-D up-regulation and promote lymphangiogenesis via c-Fos/c-Jun pathway in lung cancer.

1. Introduction

Tumour invasion and metastasis are the critical steps in determining the aggressive phenotype of human cancers. Mortality of tumour patients results mainly from cancer cells spreading to distant organs.[1] Clinical and pathological observations have shown that lymph node involvement is one of the earliest features of cancer metastasis. It strongly implicates that lymphatic vessels constitute an important pathway for dissemination of tumour cells. However, the mechanisms underlying the migration of cancer cells to regional lymph nodes or distant organs remain elusive.

Interleukin 7 (IL-7) could induce the development and proliferation of haematopoietic cells and malignancies, including some forms of leukaemia and lymphoma.[2–14] However, little was known about its involvement in solid tumours, including lung cancer. Some malignant cells, such as chronic lymphoblastic leukaemia cells,[8] Burkitt's lymphoma cells[9] and colonic cancer cells[15] were also capable of producing IL-7. Other solid tumours could express the IL-7 gene, including oesophageal,[16] renal,[17] head and neck squamous cell carcinoma,[18] and Warthin's tumour of the parotid gland.[19] In addition, expression of IL-7 and IL-7R in breast cancer tissues has been shown recently, and IL-7R was positively correlated with

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tumours that had metastasised to the regional lymph nodes.[20] Following the binding of IL-7R to its ligand, a series of intracellular phosphorylation events occurred, such as the activation of the Janus kinases (JAK-1 and JAK-3), phosphoinositide 3 kinase (PI3K), and the signal transducers and activators of transcription (STAT-5).[13] It was therefore tempting to speculate that certain unidentified downstream gene(s) of IL-7 may have a role in tumour metastasis.

Lymphangiogenesis has recently been focused in tumour lymph node metastasis due to the discovery of two major factors, VEGF-C and VEGF-D, which had been linked to inducement of lymphangiogenesis in animal models.[21,22] The binding of VEGF receptor (VEGFR)-3 and VEGF-C or VEGF-D stimulated the proliferation and migration of lymphatic endothelial cells through mitogen-activated protein kinase (MAPK) and PI3K pathway,[23] which in turn increased numbers of new lymphatic vessels. It has been shown that the high expression of VEGF-D strongly correlates with the formation of metastases in regional lymph nodes in human bladder cancer, papillary thyroid, prostate, gastric, colorectal, breast, ovarian, and lung carcinomas.[24–31] In addition, immunohistochemical staining of tumour specimens demonstrated that VEGF-D is significantly elevated in several types of tumour cells,[32] suggesting that tumour cells could generate VEGF-D, and activate lymphangiogenesis. As human VEGF-D cDNA was initially isolated using an activator protein (AP-1) overexpression strategy,[33] it was suggested that AP-1 may be directly involved in regulation of VEGF-D gene expression. Because the transcription factors and regulatory promoter elements controlling VEGF-D gene expression have not yet been identified, the role of AP-1 in the regulation of VEGF-D, however, remains hypothetical.

In this study, we studied the relationship between IL-7 and VEGF-D in human lung cancer cells and their impact on lung cancer patients' outcomes. We found that VEGF-D was one of the major downstream genes of IL-7 in lung cancer cells. The up-regulation of VEGF-D by IL-7/IL-7R was mediated by the AP-1 (c-Fos/c-Jun) pathway. IL-7/IL-7R -induced VEGF-D up-regulation correlated well with clinical stages, lymph node metastasis and survival in human non-small cell lung cancer (NSCLC) patients.

2. Materials and methods

2.1. Antibodies and reagents

Polyclonal anti-IL-7, IL-7R, VEGF-C, VEGF-D, c-Jun, phosphorylated c-Jun (p-c-Jun), and c-Fos antibodies and monoclonal anti-β-actin antibody were purchased from Santa Cruz Biotechnology (USA). Monoclonal D2-40 and CD34 antibodies and 3,3′-diaminobenzidine tetrahydrochloride (DAB) were purchased from MaiXin Biotechnology (China). Separation Columns and Protein G MicroBeads were purchased from Miltenyi Biotec (Germany). Recombinant human IL-7 was purchased from Chemicon International (USA). AP-1 inhibitor (SP600125) was purchased from Calbiochem Co. (Germany).

2.2. Cell culture

Human lung cancer cell lines A549 and SPC-A1 (adenocarcinoma), NCI-H460 and LH7 (large carcinoma), and SK-MES-1 (squamous carcinoma) were maintained in Dulbcco's Modifed Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (Gibco, USA), 100 units/ml streptomycin, and 100 units/ml penicillin in a humidified 5% v/v CO_2 atmosphere until 75% confluent. Cells were divided into three groups: IL-7 stimulating group (cells were incubated with recombinant human IL-7, (20 ng/ml)); blocking IL-7R group (cells were incubated with IL-7R specific antibody (sc-662, 100 ng/ml) and recombinant human IL-7 (20 ng/ml)); inhibiting AP-1 activity group (cells were incubated with SP600125 (AP-1 specific inhibitor 25 μmol/ml) and recombinant human IL-7 (20 ng/ml)). At 0, 1, 2, 4, 8 and 24 h incubation times with different treatment factors, cells were collected.

2.3. Western blot analysis

Total protein was extracted with lysis buffer (150 mM NaCl, 1% v/v NP-40, 0.1% v/v SDS, 2 μg/ml aprotinin, 1 mM PMSF), and 60 μg of protein lysates were separated on a 12% v/v SDS-polyacrylamide electrophoresis gel, transferred to Polyvinylidene Fluoride (PVDF) membranes. Proteins were visualised with horse-radish peroxidase-conjugated goat anti-rabbit and anti-mouse IgG (Zhongshan, Beijing, China) followed by DAB. Subsequently, densitometric analyses of the bands were performed.

2.4. RNA isolation and reverse transcriptase-PCR

Total RNA was isolated using TRIZOL (Invitrogen, USA) according to the manufacturer's instructions. The primer sequences are: VEGF-C, forward (5′-TTC CAT TAT TAG ACG TTC CCT G-3′), reverse (5′-GTG TTT TCA TCA AAT TCT CGG T-3′); VEGF-D, forward (5′-ATG AAC ATG GAC CAG TGA AGC-3′), reverse (5′-CAG CCA CCA CAT CGG AAC-3′); β-actin, forward (5′-AAA TCG TGC GTG ACA TTA A-3′) and reverse (5′-CTC GTC ATA CTC CTG CTT G-3′). The PCR products for VEGF-C (347 bp), VEGF-D (363 bp), and β-actin (513 bp) were amplified with 30 PCR cycles (1 min at 95 °C; 1 min at 53 °C, 1 min at 72 °C), and visualised by ethidium bromide staining after agarose gel electrophoresis.

2.5. Co-immunoprecipitation (CoIP)

The protein was extracted with cellular lysis buffer. Equal amounts of protein were incubated with c-Jun specific antibody immobilised onto protein G-bead for 1 h at 4 °C with gentle rotation. Beads were washed extensively with lysis buffer, boiled, and microcentrifuged. Proteins were detected with c-Fos antibody by Western blot analysis.

2.6. Chromatin immunoprecipitation (ChIP)

We performed the ChIP assay according to the instructions of the ChIP assay kit (Upstate, USA). The procedure included DNA-protein cross-linking in chromatin, shearing DNA into smaller fragments, immunoprecipitation with anti-c-Jun antibody (negative control with normal rabbit IgG), followed by PCR identification of associated DNA sequences. The PCR primer sequences designed according to VEGF-D promoter are: forward (5′-GCT GGC CTT GTT CAT CTA GC-3′) and

reverse (5′-TTT CTA GGG GTT GGC AAA TG-3′). The PCR product is 394 bp. The positive control group used total DNA.

2.7. Cell migration and invasion assays

For the migration assay, 5×10^4 cells were trypsinised, washed, resuspended in serum-free DMEM, and placed in the top portion of the chamber. The lower compartment of the chamber contained 10% v/v FBS as a chemo-attractant. The chambers were incubated at 37 °C, 5% v/v CO_2 for 6 h, then cells on the membrane were washed with PBS and fixed in 100% methanol, stained with haematoxylin, photographed, and counted. For the invasion ability assay, pre-cooled serum-free DMEM was mixed with Matrigel (BD Biosciences, USA) (1:7 dilution). The upper compartments were filled with 100 μl of the mixture, and the Matrigel was allowed to solidify at room temperature for 3 h. Other procedures followed the migration protocol (BD Biosciences, USA). The chambers were incubated for 24 h.

2.8. Patients and specimens

A total of 100 cases of NSCLC were obtained from the 1st January 1980 to the 31st December 2005 at the First Affiliated Hospital of China Medical University, Shenyang, China. The tumour tissues in this study were from patients who had NSCLC proved by pathological diagnosis without distant metastasis. None of the 100 cases had received radiation therapy or chemotherapy before surgery. The TNM staging system of the UICC (1997) was used to classify the specimens. In these cases, 59 showed lymph node metastasis (Table 1). The survival time was calculated from the operation day to death via the evaluation of recurrence and metastasis or until the last follow-up date (December 2006). The following-up of the surviving patients averaged 23.09 months and ranged from 1 to 117 months. The study has been approved by the Hospitals' Ethical Review Committee.

2.9. Immunohistochemistry and quantitation of blood and lymphatic vessel densities

Four-micron thick sections were prepared from the paraffin-embedded tissues. Immunostaining was performed by the streptavidin-peroxidase (S-P) method (Ultrasensitive™ MaiXin, Fuzhou, China). The primary antibodies were anti-IL-7, anti-IL-7R, anti-VEGF-D (1:100, 1:100, 1:150), anti-D2-40 and anti-CD34 antibodies. The peroxidase reaction was developed with DAB. For negative control, the primary antibodies were replaced by non-immune serum.

All the samples were evaluated by two independent pathologists. Intensity of immunohistochemical staining: –, negative; +, focal expression <5% of cancer tissues; ++, focal expression in 5–20% of cancer tissues; and +++, diffuse expression > 20% of cancer tissues. The section with ++ and +++ staining of IL-7, IL-7R or VEGF-D was classified as high expression and the section with – and + staining was assigned as low expression. The evaluation criteria used for determination of blood and lymphatic vessel staining were as follows: yellow-brown stained endothelial cells with band or fissure-like isolated or clustered structures or with tubular lumen

Table 1 – Relationship between IL-7 expression in non-small cell lung cancer and clinical pathological factors (Chi-square test).

	Patients	IL-7 high expression (%)	P	IL-7R high expression (%)	P
Gender					
Male	74	47 (63.51%)	0.385	47 (63.51%)	0.599
Female	26	14 (53.85%)		15 (57.69%)	
Age					
⩽60	56	36 (64.29%)	0.447	38 (67.86%)	0.173
>60	44	25 (56.82%)		24 (59.09%)	
Histology					
Squamous cancer	59	35 (74.58%)	0.68	37 (74.58%)	0.86
Adenocarcinoma	41	26 (73.17%)		25 (73.17%)	
Differentiation					
Well&Moderate	60	39 (65.00%)	0.452	39 (65.00%)	0.618
Poor	40	22 (55.00)		23 (57.50%)	
Stage					
I–II	46	20 (43.48%)	0.001*	22 (47.83%)	0.007*
III	54	41 (80.39%)		40 (78.43%)	
Lymph node metastasis					
Positive	59	45 (76.27%)	0.000*	45 (76.27%)	0.000*
Negative	41	16 (39.02%)		17 (41.46%)	
VEGF-D expression					
Positive	59	46 (77.97%)	0.000*	44 (74.58%)	0.002*
Negative	41	15 (36.59%)		18 (43.90%)	

Abbreviations: IL-7, Interleukin 7; IL-7R, IL-7 receptor; VEGF-D, vascular endothelial growth factor-D.

* $P < 0.05$.

were counted as a single blood or lymphatic vessel. Within each section, we selected three tumour areas with the highest density of distinctly highlighted microvessels and lymphatic vessels ('hot spot') when observed under low-power fields. Next, the average number of CD34- or D2-40-labelled tubular lumens was counted under high-power fields.[34] Microvessel density(MVD) = mean (CD34-labelled tubular lumen number – D2-40-labelled tubular lumen number); LVD = mean D2-40-labelled tubular lumen. If the difference between the numbers counted by the two pathologists was more than 10%, the lumens were recounted and a consensus between each observer was reached.[35]

2.10. Statistical analysis

The statistical package SPSS13.0 (SPSS incorporated, Chicago) was used for all analyses. The Chi-square test was used to determine the correlation between IL-7/IL-7R expression and clinical and pathological factors; Kaplan–Meier curves were used for survival analysis, and log-rank was determined based on the differences; Cox regression multivariate analysis was used to evaluate IL-7 and IL-7R as prognostic factors to compare them with other strong prognostic factors for prognosis in lung cancer. Other results were analysed using the *t*-test. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. IL-7/IL-7R increase the expression of VEGF-D in lung cancer cell lines

Treatment of IL-7R$^+$ lung adenocarcinoma A549 cells with recombinant human IL-7 increased the expression of VEGF-D mRNA and protein, while the expression of VEGF-C mRNA and protein was not affected (Fig. 1A and C). Interestingly, the expression of VEGF-D mRNA and protein was not affected in IL-7R$^-$ lung large carcinoma H460 cells after IL-7 stimulation. We then detected the expression of VEGF-D mRNA and protein of A549 and H460 cells after blocking IL-7R. Blockage of IL-7R in A549 cells decreased the expression of VEGF-D mRNA and protein, which was not affected by the IL-7 (Fig. 1B and C). However, blocking IL-7R in H460 cells did not affect the expression of VEGF-D mRNA and protein.

To examine whether IL-7 mediated expression of VEGF-D could be observed in other IL-7R$^+$ human lung cell lines, we next incubated SPC-A1, LH7, and SK-MES-1 cell lines, respectively, with the IL-7 or with the IL-7 and sc-662 for 24 h. We found that IL-7 increased the expression of VEGF-D protein and mRNA levels in three cells examined, and blocking IL-7R decreased the expression of VEGF-D protein and mRNA levels in all three cells (Fig. 1D and E), indicating that IL-7 could up-regulate VEGF-D via IL-7R.

3.2. IL-7/IL-7R induce VEGF-D via c-Fos/c-Jun pathway

To investigate how IL-7/IL-7R regulate VEGF-D, we checked the expression of AP-1 protein in A549 cell. Incubation of A549 cells with IL-7 increased the expression of c-Fos and c-Jun protein (Fig. 1A) while blocking IL-7R with sc-662 decreased the expressions of c-Fos, c-Jun and p-c-Jun (Fig. 1B).

To confirm whether IL-7/IL-7R up-regulate VEGF-D through activation of c-Fos/c-Jun, we then inhibited the activity of c-Fos/c-Jun with a specific AP-1 inhibitor SP600125. RT-PCR and Western blotting analyses showed that the expressions of VEGF-D mRNA and protein in A549 cells were decreased significantly after treatment with SP600125, while decreased expression of VEGF-D was not affected by the IL-7 (Fig. 1F and G). These results imply that IL-7/IL-7R up-regulate VEGF-D via c-Fos/c-Jun.

3.3. IL-7/IL-7R lead to the formation of c-Fos/c-Jun heterodimer

Since c-Fos and c-Jun could form a heterodimer to enhance its activity,[36] we explored whether IL-7/IL-7R promote the formation of c-Fos/c-Jun heterodimer. Using the CoIP approach, we found that c-Fos/c-Jun heterodimer was increased after incubation with the IL-7, and blocking IL-7R with sc-662 decreased the dimer formation, which was not affected by the IL-7 (Fig. 1H). Thus, IL-7/IL-7R could promote c-Fos and c-Jun to form a heterodimer.

3.4. IL-7/IL-7R enhance the DNA binding activity of AP-1 to the promoter of VEGF-D

AP-1 is a transcription factor that binds to the promotor of a specific target gene to regulate its transcription. To examine whether AP-1 binds to VEGF-D promoter, we first analysed the promoter sequence of VEGF-D gene (GenBank accession no. AY874421) and identified AP-1 binding sites (consensus sequence 5′-TGAG/CTCA-3′), also known as TPA-responsive elements (TREs). We then performed ChIP assay. ChIP analysis demonstrated that AP-1 could bind to the VEGF-D promoter. We then detected AP-1 DNA binding activity in the A549 cell after incubation with either the IL-7 with or without sc-662. IL-7 enhanced AP-1 binding to the VEGF-D promoter, while blocking IL-7R reduced AP-1 binding to the VEGF-D promoter (Fig. 1I).

Migration and invasion assays showed that neither IL-7 nor sc-662 affects the number of A549 cells through the microporous membrane.

3.5. Expression of IL-7/IL-7R correlates with clinical stages, VEGF-D level, lymphatic vessel density, lymph node metastasis, and NSCLC patient survival

Immunohistochemical analysis of 100 NSCLC specimens revealed that the expression of VEGF-D was significantly associated with the expression of IL-7 and IL-7R (Table 1). In addition, the expression of IL-7 in tumours was strongly associated with the expression of IL-7R in tumours ($P < 0.001$). We then quantified the number of blood vessels and lymphatic vessels in NSCLC with high and low expression of IL-7, IL-7R and VEGF-D in NSCLC using antibodies against CD34 and D2-40, respectively. These intratumoural blood vessels and lymphatic vessels exhibited a distinctive reticular architecture with antibodies to CD34 and D2-40 staining in tumours. Quantification of the D2-40-positive vessels in the tumours revealed that tumours with increased expression of IL-7 associated with a higher LVD (15.1–44.58, $n = 61$), and low IL-7

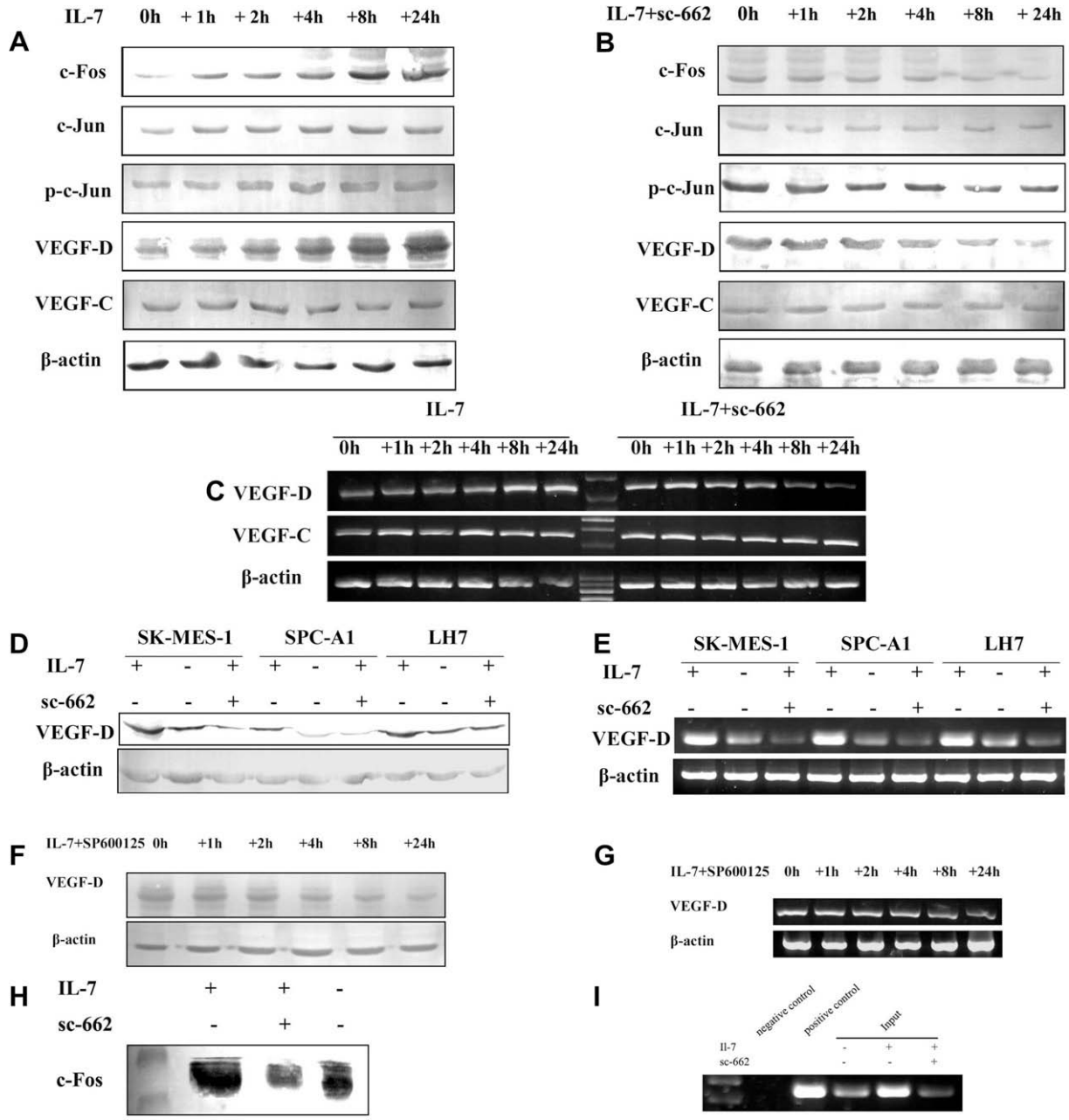

Fig. 1 – IL-7 stimulating VEGF-D expression via AP-1. Western blot analysis showing up-regulation of c-Fos, c-Jun, p-c-Jun, and VEGF-D proteins in A549 cells treated with IL-7 (A), and blocking IL-7R with IL-7R specific antibody (sc-662) downregulating the expression of c-Fos, c-Jun, p-c-Jun, and VEGF-D proteins (B). The RT-PCR analysis showing induction of VEGF-D mRNA after Il-7 stimulation, and reduction of VEGF-D mRNA after blocking IL-7R (C). The VEGF-D protein and mRNA expression in IL-7R$^+$ lung cancer cell lines (D, E). Effect of AP-1 inhibitor (SP600125) on the expressions of VEGF-D protein and mRNA (F, G). IL-7 could induce the formation of AP-1 (c-Fos/c-Jun) heterodimmer via IL-7R with CoIP method (H). The ChIP analysis showing that AP-1 (c-Fos/c-Jun) could bind with VEGF-D promoter, and the activity of binding was enhanced by IL-7/IL-7R (I).

expressing tumours showed a low level of LVD (10.35–32.69, $n = 39$). There was a significant association between the expression of IL-7 and the level of LVD ($P = 0.003$). In addition, tumours with high and low expression of IL-7R showed 13.9–43.76 ($n = 62$) and 11.31–34.59 ($n = 38$) of LVD, respectively ($P = 0.041$). Moreover, tumours with high and low expression of VEGF-D displayed 21.76–45.68 ($n = 59$) and 6.56–26.1 ($n = 41$) of LVD, respectively ($P < 0.001$). These results indicated that the expression patterns of IL-7, IL-7R, and VEGF-D associated well with the expression of LVD in NSCLC tissues (Fig. 2). Quantification of the CD-34-positive minus the D2-40-positive vessels in the tumours indicated that expressions level of IL-7, IL-7R, and VEGF-D in tumours did not correlate with MVD.

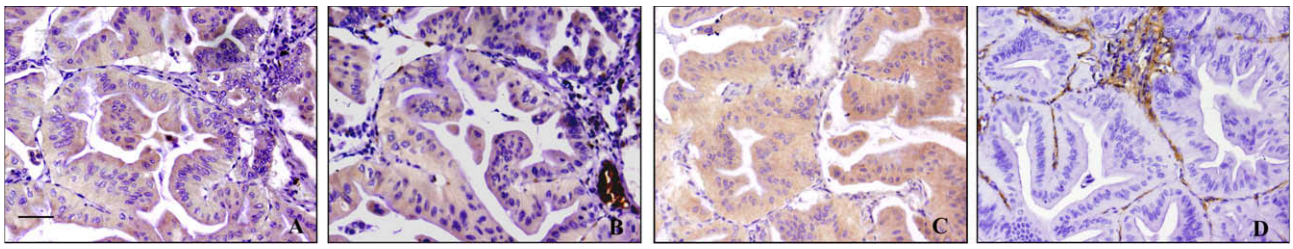

Fig. 2 – The expressions of IL-7, IL-7R, and VEGF-D in NSCLC correlate with the level of LVD. Immunohistochemical staining of consecutive serial sections of NSCLC tissues. Brown–yellow particles of IL-7 (A), IL-7R (B), and VEGF-D (C) were observed in cancer cells. When the expression level of these molecules was high, the corresponding D2-40-labelled LVD (D) were also high (400 × magnification. Scale bar 50 μm).

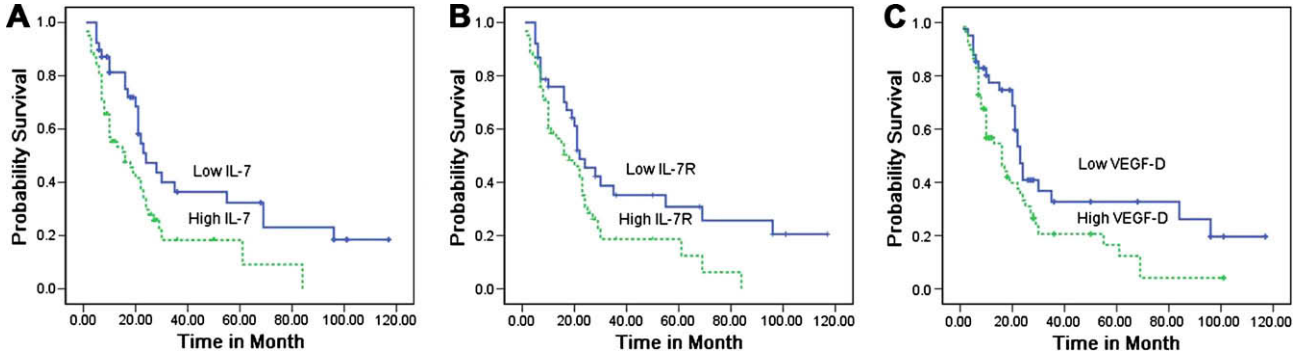

Fig. 3 – Survival of NSCLC patients correlates with the expression of IL-7, IL-7R, and VEGF-D. Kaplan–Meier survival plots for patients with NSCLC, grouped according to IL-7 (A), IL-7R (B) and VEGF-D (C) protein expression. Correlation between overall survival of patients with IL-7/IL-7R expression were found to be statistically significant ($P = 0.008$ and $P = 0.031$, respectively) as was that between survival rate and VEGF-D expression ($P = 0.022$). All patients alive at their last follow-up are indicated by tick marks on the plot.

Clinically, compared to the NSCLC with low expression of IL-7, tumours with high expressions of IL-7, IL-7R, and VEGF-D were more advanced, with lymph node metastasis ($P < 0.01$). Patients with low expression of IL-7 had a statistically significant longer survival than those with high expression of IL-7 (median survival = 16 ± 4.5 months; 95% confidence interval 7.1–24.9 months; $P = 0.008$; Fig. 3A). In addition, patients with low expression of IL-7R survived longer than those with high expression of IL-7R (median survival = 18 ± 4.1 months; 95% confidence interval 10.0–26.0 months; $P = 0.031$; Fig. 3B). The median survival time for NSCLC patients with high expression of VEGF-D was 16 ± 3.8 months; 95% confidence interval 8.6–23.4 months, shorter than those with low expression of VEGF-D ($P = 0.022$, Fig. 3C). In the Cox regression multivariate analysis, lymph node metastasis and tumour stage were the strongest predictors of survival (Table 2). These results provide a possible explanation that IL-7 and IL-7R high expression patients were more likely to have poor prognosis, possibly resulting from IL-7/IL-7R-mediated lymphangiogenesis via up-regulation of VEGF-D in human NSCLC.

4. Discussion

IL-7 is produced mainly by thymus,[37,38] bone marrow, intestinal epithelium[39] and skin.[40] The IL-7 ligand receptor system plays an important role in the normal development and maintenance of the human immune system. In breast cancer cell lines, IL-7 could up-regulate expression of VEGF-D,[41] while this effect involved PI3K.[42] Furthermore, IL-7 has been identified as a putative lymphangiogenic factor in human endothelial cells,[43] suggesting a possible role of IL-7 in tumour lymphangiogenesis and metastasis.

In the present study, we reported that recombinant human IL-7 increases the expression of VEGF-D mRNA and protein in lung cancer cell lines, and blocking IL-7R could abolish the role of IL-7 in VEGF-D. In addition, we found that AP-1 could bind to VEGF-D promoter. Moreover, IL-7 induced c-Fos, c-Jun expression and phosphorylation, promoted c-Fos and c-Jun heterodimer formation, and enhanced the activity of c-Fos/c-Jun. Consistent with our finding, it has been shown that IL-7 increases IL- 2-gene expression via regulating AP-1 DNA binding activities in activated human T lymphocytes.[44]

AP-1 is a group of structurally and functionally related members of the Jun protein family [Jun (originally described as c-Jun), JunB and JunD] and Fos protein family [Fos (originally described as c-Fos), FosB, Fra-1 and Fra-2]. A common feature of all these proteins is the evolutionarily conserved bZIP domain, the collective term for a basic DNA binding domain combined with a leucine zipper region. The leucine zipper is responsible for dimerisation, which is a prerequisite for DNA binding mediated by the basic domain. The composition

Table 2 – Multivariate Cox regression model.

	Wald	Exp(B)	95% CI for Exp(B)	P
Gender	0.703	1.315	0.693–2.494	0.402
Age	1.899	0.696	0.416–1.165	0.168
Histology	2.752	1.587	0.920–2.738	0.097
Differentiation				
Well	1.367			0.505
Moderate	1.205	0.643	0.292–1.415	0.272
Poor	0.000	0.995	0.553–1.793	0.988
Stage				
I	25.280			0.000*
II	23.143	0.015	0.003–0.082	0.000*
III	5.556	0.266	0.088–0.800	0.018*
Lymph node metastasis	7.692	8.473	1.871–38.358	0.006*
IL-7 expression	1.785	0.562	0.241–1.309	0.182
IL-7R expression	0.732	1.409	0.642–3.092	0.392
VEGF-D expression	2.074	0.603	0.303–1.200	0.150
LVD	0.346	0.993	0.968–1.018	0.557

Abbreviations: IL-7, Interleukin 7; IL-7R, IL-7 receptor; VEGF-D, vascular endothelial growth factor-D; LVD, lymphatic vessels density.
* $P < 0.05$.

of the leucine zipper is also responsible for the specificity and the stability of homo- and heterodimers formed by the various Jun and Fos proteins. Whereas the Jun proteins exist as homo- and heterodimers, the Fos proteins, which cannot homodimerise, form stable heterodimers with Jun proteins and thereby enhance their DNA binding activity.[36]

It has been shown that a few colon cancer cells could produce IL-7, breast cancer tissues express IL-7 and IL-7R, and IL-7R positively correlates with tumours metastasised to the regional lymph nodes.[20] In this study, we further evaluated expression of IL-7 and IL-7R in 100 NSCLC tissues. Interestingly, we have found that the high expression of IL-7 and IL-7R correlated well with high expression of VEGF-D, clinical stages, higher lymphatic vessel density, more lymph node metastasis, and a shorter survival rate of patients. Together with our molecular studies in lung cancer cells, it is likely that IL-7/IL-7R promote lymphangiogenesis via VEGF-D, which in turn induces lymph node metastasis in NSCLC tissues.

In conclusion, this study demonstrated that, in human lung cancer cells, IL-7 and its receptor IL-7R, are able to induce VEGF-D gene expression via AP-1 (c-Fos/c-Jun)-dependent pathway. *In situ* analysis of human NSCLC revealed that overexpression of IL-7/IL-7R and VEGF-D play an important role in lung cancer lymphangiogenesis. Thus, targeting IL-7/IL-7R may potentiate new therapeutic strategy against lymphangiogenesis and lymphatic node metastasis.

Conflict of interest statement

None declared.

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