# c-Jun, a crucial molecule in metastasis of breast cancer and potential target for biotherapy

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**Abstract.** Human breast cancer cell line SKBR3 expresses high level of the ErbB2 molecule, which has been associated with poor prognosis of breast cancer. Elevated ErbB2 functions as a transactivating co-receptor and promotes the formation of ErbB2 containing heterodimers, which are more mitogenic and transforming, and have a higher ligand affinity and signaling potency by virtue of the potent latent kinase activity of ErbB2. Interestingly, SKBR3 cells are non-tumorigenic in nude mice. By ectopic overexpression of c-Jun in SKBR3 cells, involvement of c-Jun in invasiveness and metastasis *in vivo* was investigated in this study. The critical role of c-Jun in the tumorigenesis and metastasis is demonstrated in nude mice.

## Introduction

The transcription factor AP-1 consists of two family members, Fos and Jun. It regulates a large variety of biological processes including proliferation, differentiation, apoptosis and oncogenic transformation, and contributes to both basal and stimulus-activated gene expression (1,2). The Jun proteins (c-Jun, JunB, JunD) can form homodimers, but the Fos proteins (c-Fos, FosB, Fra-1, Fra-2) must heterodimerize with Jun members to form transcriptionally active complexes (3). *In vitro* studies have shown that Jun/Fos heterodimers are more stable and have a higher DNA binding activity than Jun homodimers. The activity of AP-1 transcription factor complex is modulated by growth factors, proinflammatory cytokines, UV radiation, and tumor promoters such as the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA). Whereas, the extracellular signals are converted into changes of the expression of specific target genes, which harbor AP-1 binding sites, the specific DNA sequences called TPA response elements (TREs) in their regulatory regions (4).

Among AP-1 complexes, c-Jun is the major component. Previous studies have implicated that c-Jun is important in breast tumorigenesis. c-Jun/AP-1 associates with invasive properties of aggressive breast cancer. A recent study showed that the overexpression of c-Jun in the breast cancer cell line MCF-7 resulted in morphological changes, increased mobility and invasion *in vitro*, and tumor formation *in vivo* (5). The cells expressing dominant-negative c-Jun fail to invade in response to EGF (6). Whether c-Jun over-expression can cause tumor metastasis *in vivo* has not been demonstrated.

Human breast cancer cell line SKBR3 expresses high level of the ErbB2 molecule, which has been associated with poor prognosis of breast cancer. Elevated ErbB2 functions as a transactivating co-receptor and promotes the formation of ErbB2 containing heterodimers, which are more mitogenic and transforming, and have a higher ligand affinity and signaling potency by virtue of the potent latent kinase activity of ErbB2 (7,8). Interestingly, SKBR3 cells are non-tumorigenic in nude mice. By ectopic overexpression of c-Jun in SKBR3 cells, involvement of c-Jun in invasiveness and metastasis *in vivo* was investigated in this study. The critical role of c-Jun in the tumorigenesis and metastasis is demonstrated in nude mice.

## Materials and methods

*Breast cancer cell lines*. Human breast cancer cell lines MCF-7, SKBR3, T47D, MDA231 and MDA435 were obtained from American Type Tissue Culture Collection (Rockville, MD) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FCS), 1 mM glutamine and antibiotics.

*Construct and transfection*. Full-length c-Jun cDNA was amplified by reverse transcription PCR (RT-PCR) with LA TaqDNA polymerase (Takara) and the specific primers (Table I). The resulting fragment was cloned into the plasmid pcDNA3.1 (Invitrogen) designated pcDNA3.1/c-Jun. SKBR3 cells were transfected with pcDNA3.1/c-Jun by using Lipofectamin PLUS reagent (Gibco BRL). The empty vector

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Table I. Primers used in RT-PCR analysis.

| Gene | Primer pairs | $T_m$ | Cycles | Length of amplicons (bp) |
|---|---|---|---|---|
| *c-Jun* | 5'-GGAATTCCACCATGACTGCAAAGATGGAAACGACCT-3'<br>5'-CGGGATCCCGTTATCAAAATGTTTGCAACTGCTGCGTT-3' | 60 | 32 | 996 |
| *eIF-4E* | 5'-ACCCCTACTCCTAATCCCCCGA-3'<br>5'-GGGGCAAACAAAAAGAGTAGAA-3' | 53 | 26 | 305 |
| *VEGF* | 5'-AACTTTCTGCTGTCTTGGGTG-3'<br>5'-TGGCCTTGGTGAGGTTTGAT-3' | 56 | 26 | 341 |
| *CD44v6* | 5'-CAGGCAACTCCTAGTAGT-3'<br>5'-AGCTGTCCCTGTTGTCGA-3' | 53 | 26 | 126 |
| *CXCR4* | 5'-GAAAAGGTGGTCTATGTTGGCG-3'<br>5'-TCCTACTGACACCAGAACTCCC-3' | 58 | 28 | 275 |
| *GAPDH* | 5'-GGTCGGAGTCAACGGATTTG-3'<br>5'-ATGAGCCCCAGCCTTCTCCAT-3' | 55 | 28 | 350 |

Forward primer is shown as upper sequence in the respective primer pair. Reverse primer is the lower sequence.

was used as a control. To obtain transfectants stably expressing c-Jun, the transfected SKBR3 cells were selected in the presence of G418 (Sigma) at a concentration of 800 $\mu$g/ml.

*RT-PCR*. Total-RNA was isolated from SKBR3 cells using the TRIzol reagents (Gibco BRL) and quantified by spectro-photometry. Reverse transcription was performed. The resulting single stranded cDNA was subsequently amplified by using specific primers (Table I). Amplification of glycer-aldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal control. The intensity of bands was quantified using LabWorks™ Image Acquisition and Analysis software (Ultra-Violet Products Ltd., Cambridge, UK). The experiment was conducted in triplicate.

*Cell lysate preparation and Western blot*. The cells were lysed in ice-cold lysis buffer [50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1 x cocktail protein inhibitors (Roche)]. The lysates were fractionated by SDS-PAGE, blotted on nitrocellulose membranes and detected with anti-c-Jun antibody (Santa Cruz) and horseradish peroxidase-conjugated secondary antibodies (Beijing Zhongshan Co.). The bands were visualized by SuperSignal® West Femto Maximum Sensitivity Substrate (Pierce).

*In vivo studies*. Five-week-old female Balb/C athymic nude mice were purchased from the Institute of Laboratory Animal Science, Chinese Academy of Medical Science and housed in specific pathogen-free conditions in Good Laboratory Practice facility. SKBR3 or SKBR3/c-Jun cells (2x10⁷) were injected s.c. in the right flank. For experimental liver metastasis assays, mice were injected intravenously with 3x10⁶ tumor cells via the lateral tail vein. Primary tumors and livers were autopsied 20-30 days post injection and tissues with metastases

were either photographed for gross morphology or processed for histology and immunohistochemistry.

*Histology and immunohistochemistry*. Tissues were dissected and fixed in 10% buffered formalin and embedded in paraffin wax. Tissue sections were stained with H&E for morpho-logy. The photographs were taken on a Nikon microscope.

For immunohistochemistry study, the sections were dewaxed with xylene, and gradually hydrated in a decreasing ethanol series. Endogenous peroxidase activity was quenched using 3% hydrogen peroxide in distilled water and then washed in phosphate-buffered saline (PBS). After antigen retrieval, the sections were incubated with the rabbit antibody against c-erbB-2 (LAB Vision, USA) and subsequently with horseradish peroxidase-conjugated anti-rabbit antibody (Gene Tech Biotechnology Co.) after washing with PBS. The color was developed by incubation with 3,3'-diaminobenzidine (DAB) solution. Omission of the primary antibody and substitution by non-specific immunoglobulin at the same concentration were used as negative controls.

*Statistical analysis*. Data were expressed as mean ± SD. Data were compared by the Student's t-test. P<0.05 was considered statistically significant.

**Results**

*Expression of c-Jun in breast cancer cell lines*. A number of widely available human breast cancer cell lines have been tested for their tumorigenesis and metastatic property. To evaluate the correlation of c-Jun expression with tumori-genesis, in this study, five different human breast cancer cell lines were utilized to test c-Jun expression at protein level. Both MDA231 and MDA435 cell lines are identified as
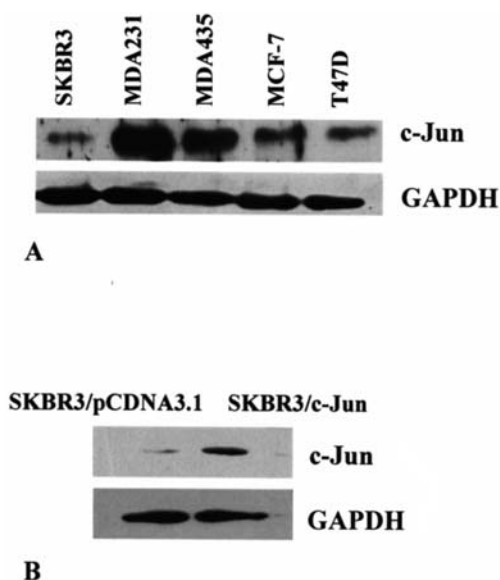
Figure 1. Western blot analysis. Expression of c-Jun in breast cancer cell lines (A) and transfected SKBR3 cells (B).

highly tumorigenic and metastatic (9,10), producing lung and lymph node metastases in a high proportion of nude and severe combined immunodeficient (SCID) mice after injection in the mammary fatpad or after tail vein administration. Whereas, SKBR3 cells are non-tumorigenic in nude mice (11,12). MCF-7 and T47D cell lines require estrogenic supplementation for tumorigenesis in nude mice (13). To compare c-Jun expression in various breast cancer cell lines, Western blot was performed using the commercial mono-

clonal antibody recognized c-Jun. The high protein level of c-Jun with a specific band of 39 kDa was detected in MDA231 and MDA435 cells (Fig. 1A). Faint bands were also identified in MCF-7 and T47D cells representing a very marginal presence of c-Jun. However, c-Jun expression in SKBR3 cells was the lowest.

In order to investigate the effects of c-Jun on tumorigenesis and metastasis of breast cancer cells, pcDNA3.1/c-Jun was transfected into SKBR3 cells. The transfected SKBR3 cells were selected in G418 for stable c-Jun expression. Individual neomycin-resistant colonies (SKBR3/c-Jun) from pcDNA3.1/c-Jun transfections were isolated and expanded. Western blot analysis showed that c-Jun protein level was increased in the SKBR3 stable transfectant (Fig. 1B).

*Effect of c-Jun expression on potential of SKBR3 tumor formation and liver metastases in nude mice.* The effect of c-Jun expression on tumor formation in nude mice was investigated by injecting $2x10^7$ cells subcutaneously into mice. The growth of SKBR3/c-Jun tumors was observed in mice after injection for 5-7 days. Histologically, the tumors were composed of relatively large cells with striking heteromorphism and mitosis (Fig. 2A). Then the effect of c-Jun on the metastatic potential was investigated by tail vein injection. Metastatic colonization was evaluated by gross examination and microscopic inspection of tissue sections. The results showed that livers with SKBR3/c-Jun cell derived metastases were enlarged. Visible metastatic lesions of livers were observed within 20 days by gross examination (Fig. 2B). Microscopic examination of liver tissue sections revealed a massive infiltration of the liver by SKBR3/c-Jun derived tumor cells (Fig. 2C and D). The size of tumor cells metastasizing
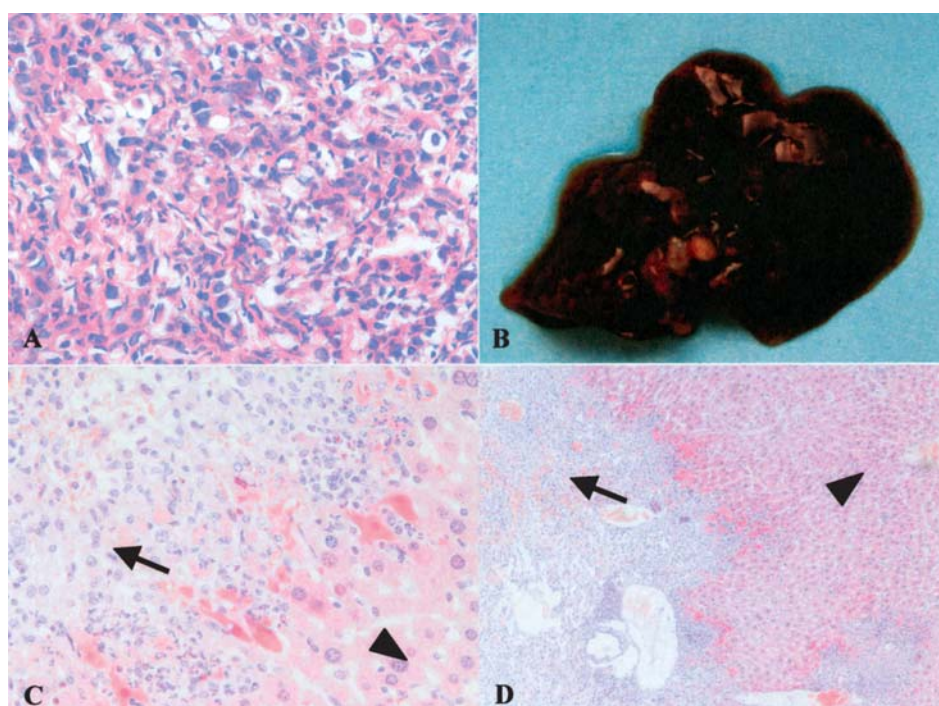


Figure 2. Histological observation. (A) SKBR3/c-Jun derived tumor tissue; (B) Gross examination of livers with SKBR3/c-Jun cell derived metastases; (C and D) The massive infiltration of the liver by SKBR3/c-Jun derived tumor cells (C, higher magnification; D, lower magnification). Arrow, SKBR3/c-Jun cell derived metastases; triangular arrow, liver tissue.
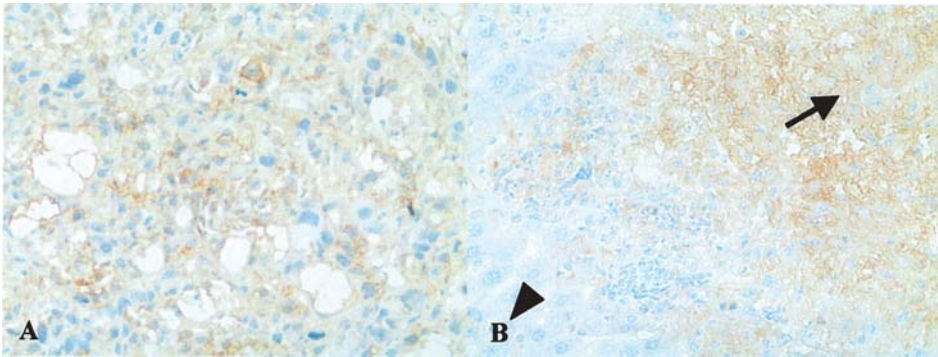
Figure 3. Immunohistochemical analysis. (A) SKBR3/c-Jun derived tumor tissue; (B) SKBR3/c-Jun cell derived metastases. Arrow, SKBR3/c-Jun cell derived metastases; triangular arrow, liver tissue.
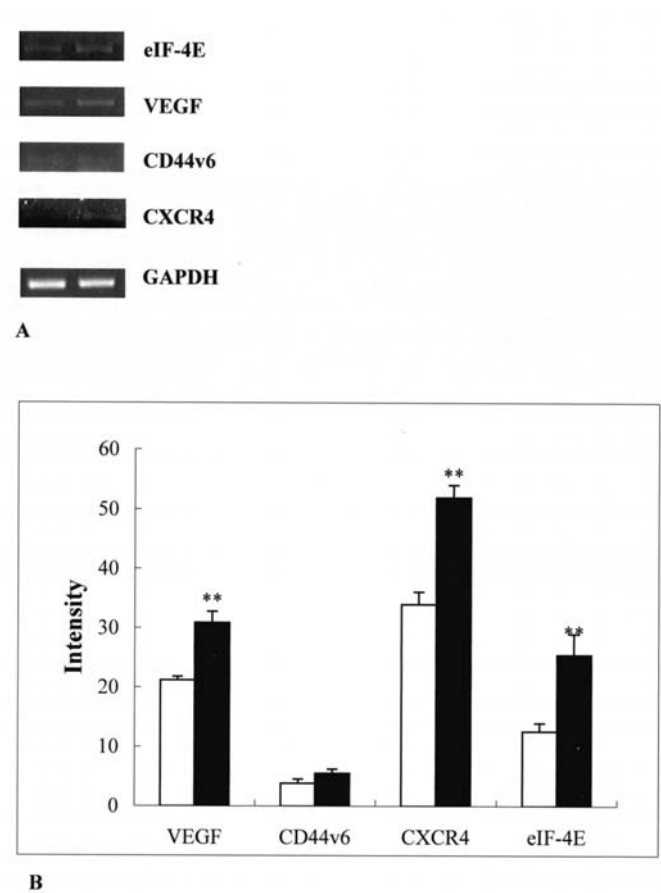


Figure 4. Effect of c-Jun overexpression on the transcription of genes related to metastasis. (A) The expressions of *CXCR4*, *eIF-4E*, *VEGF* and *CD44v6* at mRNA level analyzed by semi-quantitative RT-PCR. (B) The intensity of bands was quantified using LabWorks™ Image Acquisition and Analysis software. Empty column, SKBR3 cells transfected with pcDNA3.1; solid column, SKBR3 cells transfected with pcDNA3.1/c-Jun.

to the livers appeared smaller than that from subcutaneous tumors. Neither tumors nor metastatic nodules of livers were found in the mice injected with parental SKBR3 cells.

To confirm the origin of the heterologous cells in the nude mice liver, immunohistochemical staining was performed on the paraffin sections. The results clearly demonstrated that the cells from both the subcutaneous tumor tissues and metastasis in the livers of the mice were specifically labeled by the antibody against human ErbB2 (Fig. 3), indicating that SKBR3/c-Jun derived cells formed tumors, and metastasized to the liver.

*Effect of c-Jun expression on the transcription of genes related to metastasis.* Previous studies have indicated an association of overexpression of *CXCR4*, *VEGF*, *eIF4E* and *CD44* (or alternatively spliced variants) with aggressiveness of a variety of human tumors. The promoter regions of these genes contain putative binding sites for AP-1. The expressions of the genes at mRNA level were studied by semi-quantitative RT-PCR. As shown in Fig. 4, the up-regulation of these genes in SKBR3/c-Jun cells was observed compared to the mock transfected SKBR3 cells.

## Discussion

The experimental studies showed that stronger proliferation, malignant transformation and enhanced aggressiveness were accompanied by changes in AP-1 complex composition. The influence of the AP-1 on the transcription of target genes is dependent on the abundance of single Jun and Fos proteins as well as the specific binding sequences (14). c-Jun is a prominent component of many AP-1 complexes. Previous studies and our unpublished data demonstrate that ectopic overexpression of c-Jun in MCF-7 breast cancer cells results in increased motility and invasiveness, indicating that c-Jun may play an very important role in the progress of breast cancer.

In the present study, the effect of c-Jun on the metastasis of breast cancer was investigated by introducing c-Jun into SKBR3 cells. Among the breast cancer cell lines tested, SKBR3 cells express the highest level of ErbB2 (15,16), a well-known oncoprotein. Due to its unusual genetic background SKBR3 cells are not capable of forming tumors in nude mice. Notably, c-Jun expression in SKBR3 cells was the lowest among five breast cancer cells tested. By ectopic expression of c-Jun in SKBR3 cells, surprisingly, the SKBR3/c-Jun xenograft tumor was established in nude mice. Furthermore, the introduction of c-Jun gene alone resulted in transfected cells capable of metastasizing to the nude mouse liver. To our knowledge, this is the first report providing evidence that c-Jun expression induced SKBR3 derived tumor liver metastasis in nude mice.

AP-1 is activated by extracellular stimuli through various signal transduction pathways, predominantly the mitogen-activated protein kinase (MAPK) cascade. MAPK tyrosine and threonine phosphorylation at the final level of the cascade activates three MAPK family members, extracellular signal-regulated protein kinase (ERK), c-Jun amino-terminal kinase (JNK), and the high osmolarity glycerol response kinase (p38 $\alpha, \beta, \gamma, \delta$). AP-1 can be activated by all three of the MAPK pathways (17). c-Jun is a nuclear substrate of JNK1 and its transactivation activity is augmented by amino (N)-terminal phosphorylation at serines 63 and 73 (18,19). Oncogenic ErbB2 can increase AP-1-dependent gene expression (20). Thus, ErbB2 signaling pathway ligands, ErbB receptors and their relevant intracellular transduction molecules (ras, raf-1, MAPK, JNK and p38), as well as AP-1 components together exert positive or negative influences on the expression of target genes (21). c-Jun/AP-1 participated as a signal responsive transcription factor complex at the end of the signaling cascades, providing a missing link between growth factor signaling and cellular effects, and ultimately contributing to a diversity of important end-points, including oncogenic cell transformation, tumor cell invasiveness, angiogenesis and metastasis. In this study, the changes of ERK and JNK expression and phosphorylation were not detected (data not shown). However, by introducing a single c-Jun gene into SKBR3 cells and compensating for defective c-Jun protein expression, non-tumorigenic SKBR3 cells were consequently conferred the capability of tumor formation and liver metastasis *in vivo*, strongly demonstrating that c-Jun is a determinant factor in the development of breast tumor and metastasis. The data suggest that c-Jun might be considered as an important target for breast cancer therapy.

Tumor progression towards the malignant phenotype requires expression changes of genes, many of which are involved in proliferation (cyclin D1, Rb, p16) or tumor invasion (MMPs, uPA, PAI-1, etc.) and have been found to be regulated by AP-1 (22,23). By a cDNA microarray analysis of 588 genes, the differential expression of 21 genes, which were either up-regulated or down-regulated by c-Jun, was identified in breast cancer (24). To investigate the changes of transcription of invasion-associated genes, several reported AP-1-dependent genes including *CXCR4* (25), *VEGF* (26), *CD44* (27), and *eIF4E* (28) were analyzed by RT-PCR. Compared with the parental SKBR3 cells, a tendency of the transcription up-regulation of the genes was noted in the SKBR3/c-Jun cells. However, the role of c-Jun in the process of tumorigenesis and in particular their involvement in the oncogenesis and metastasis is still not clear.

Collectively, the present study confirms that c-Jun as an ending molecule of signal transduction pathways influences the biological behavior of breast cancer cells. Ectopic expression of c-Jun is involved in the metastasis of breast cancer.

## Acknowledgements

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