SPANDIDOS PUBLICATIONS

Oncogenic properties of a novel gene *JK-1* located in chromosome 5p and its overexpression in human esophageal squamous cell carcinoma

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Abstract. Esophageal squamous cell carcinoma (ESCC) shows high frequency and mortality in Asian regions, including China. Previous analysis of genomic DNA of ESCC using comparative genomic hybridization indicated that amplification of the chromosome 5p regions is a common event in ESCC cell lines and patient cases of Hong Kong Chinese origin, and the results suggested that the genes located in the chromosome 5p regions may play crucial roles in the molecular pathogenesis of ESCC. Our previous studies on ESCC confirmed the tumorigenic and overexpression properties of a novel gene *JS-1* located in chromosome 5p15.2 upstream to δ-catenin. In the present study, another novel gene *JK-1* which is located at 5p15.1 downstream to δ-catenin was characterized for its roles in the pathogenesis of ESCC. Thirteen ESCC cell lines and 30 surgical specimens of esophageal tumors were studied for the overexpression of *JK-1* using multiplex RT-PCR analysis. The transforming capacity of overexpression of *JK-1* was also investigated by transfecting NIH 3T3 and HEK 293 cells with the expression vector cloned with *JK-1*, followed by the soft agar and foci formation assays. *JK-1* was overexpressed in 9/13 (69%) of the ESCC cell lines and 9/30 (30%) of the ESCC patient cases. Both NIH 3T3 and HEK 293 cells acquired the properties of anchorage-dependent and -independent growth when *JK-1* was overexpressed. Most significantly, subcutaneous sarcomas were formed in all (3/3) the athymic nude mice after NIH 3T3 cells overexpressing *JK-1* were injected subcutaneously. Our results thus indicated that *JK-1* is commonly overexpressed in ESCC and has a prominent capacity to transform normal cells. Our overall results thus provide the first evidence that the overexpression of *JK-1* and its transforming capacity in normal cells may play a critical role in the molecular pathogenesis of ESCC.

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Key words: esophageal squamous cell carcinoma, gene overexpression, transforming capacity, tumorigenicity

Introduction

Esophageal squamous cell carcinoma (ESCC) has a high mortality rate, ranked as the 8th most fatal cancer in Hong Kong in 2004 (Department of Health, 2004). Previous results of comparative genomic hybridization (CGH) showed a strong signal of genomic amplification of the chromosome 5p regions in some ESCC cell lines (1) and patient cases (2) of Hong Kong Chinese origin. This observation implied that the genes on the region of chromosome 5p play crucial roles in the pathogenesis of ESCC. Gene amplification was regarded as one of the major genomic aberrations and a common mechanism for oncogene overexpression and/or for activating proto-oncogenes (3). It was observed that the amplified DNA might include some critical genes whose overexpression provided a selective force for tumor development (4). However, according to published data of the Human Genome Project, genes located at the region of chromosome 5p are largely novel to us and their functions are unknown. At present, investigations involving chromosome 5p are limited. A known gene located within the 5p contig is *δ-catenin* (CTNND2) which was reported to be relevant in tumor progression (5). Two novel genes, designated as *JS-1* and *JS-2*, have been investigated by our group previously for their roles in the pathogenesis of ESCC (6). They are mapped to 5p15.2

and located at the 5' upstream region of CTNND2. *JS-1* was confirmed to have a prominent transforming capacity in normal mouse fibroblast cells (6). In this report, another novel gene located in 5p15.1 (*JK-1*), which is 3' downstream to CTNND2 (Fig. 1), was characterized for its roles in the molecular pathogenesis in ESCC. The sequence of *JK-1* has been published by the National Center for Biotechnology Information (NCBI) in the category of the 'Human Genome Project' (accession no. NM_019000). Collectively, our recent studies on these ESCC-related genes may explain the significance of the overexpression of the novel candidate genes located in the chromosome 5p region on ESCC development and/or progression and prognosis.

Materials and methods

Cell lines and tissue specimens. Four ESCC cell lines of Hong Kong Chinese origin, including SLMT-1 (1), HKESC-1 (7), HKESC-2 and HKESC-3 (8) were kindly provided by Professor Gopesh Srivastava of the Department of Pathology, The University of Hong Kong. These 4 cell lines were maintained as described (6). Nine ESCC cell lines of Japanese origin, namely KYSE 30, 70, 140, 150, 180, 410, 450, 510 and 520 (9), were purchased from DSMZ (Braunschweig, Germany) and were cultured as described (9). NE1 cells are non-tumor esophageal epithelial cells immortalized by the induction of genes E6 E7 of human papillomavirus type 18 (10). These cells were kindly provided by Professor George S.W. Tsao from the Department of Anatomy, The University of Hong Kong and were cultured as described (10). Thirty fresh specimens of patient esophageal tumor tissues together with their corresponding non-tumor esophageal epithelial tissue were collected after esophagectomy at the Department of Surgery, Queen Mary Hospital, Hong Kong from 1990 to 2001. The histopathological features were reported by the Department of Pathology, Queen Mary Hospital, Hong Kong (Table I).

RT-PCR analysis. The extraction of total RNA and reverse transcription were performed as previously described by our group (6). The expression level of *JK-1* (accession no. NM_019000) in the cell lines and patient specimens of esophageal tumors was analyzed by RT-PCR analysis. cDNA (~2 μg) produced by reverse transcription from the RNA of ESCC cell lines, NE1 cells, tumors and their corresponding non-tumor tissues was amplified as previously described by using a specific PCR primer pair for the *JK-1* gene and the specific glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene primers acting as an internal control for normalizing the cDNA quantity. Primers for *JK-1* were: 9000-r, 5'-TTC CAA ACC AGA TGA AAG AC-3' and 9000-t, 5'-CGG TCA AGA TCA TCA GAA GT-3'; and primers for *GAPDH* were: *GAPDH-1,* 5'-TGA AGG TCG GAG TCA ACG GAT TTG GT-3' and *GAPDH-2*, 5'-CAT GTG GGC CAT GAG GTC CAC CAC-3' (Clontech). The PCR product was then electrophoresed in a 2% agarose gel and visualized under ultraviolet. The intensities of the PCR products were measured by densitometric analysis using the Quantity One program (Bio-Rad). The intensities of the target PCR product were normalized against the *GAPDH* PCR product of each sample. The relative level of *JK-1* mRNA expression was expressed by the formula: (JK-1/GAPDH, tumor)/(JK-1/GAPDH, non-tumor) (11). The expression was regarded as overexpression when the (JK-1/ GAPDH, tumor)/(JK-1/ GAPDH, non-tumor) ratio was >1.2; a ratio between 0.8 and 1.2 was regarded as no change, and a ratio <0.8 was regarded as underexpression of the gene (12).

Cloning of JK-1. The coding sequence of *JK-1* was produced from the cDNA of NE1 cells by PCR amplification using the primers 9000-a, 5'-ATT AGC CGT GGT TAG CAG GT-3' and 9000-d, 5'-TCC AAT TAA TTC ACT GCA GGA GG-3'; followed by a nested PCR using primers 9000-b, 5'-GCA GAA ATG CCT GAA GGT GAA G-3' and 9000-c, 5'-TCT GTT GCA AGC TGA TTC CTA GA-3' (Clontech). The PCR products were purified by using QIAEX II gel extraction system (Qiagen) and cloned into pGEM-T easy vector (Promega), and then subcloned into pcDNA3.1(-) expression vector (Invitrogen) according to the manufacturer's protocol. DNA sequencing of the extracted plasmid was performed to confirm the sequence identity and correct orientation of the cloned region as described previously (6).

Transformation study. Approximately 1 μg of pcDNA3.1-*JK-1* construct, mock vector pcDNA3.1(-) and pcDNA3.1-H-rasV12 construct (13) was used to transfect NIH 3T3 and HEK 293 cells separately ($1x10^5$ cells were seeded into each well of a 6-well plate). Transfection was performed as previously described (6). The *JK-1*-transfected cells were harvested by trypsinization and subjected to RT-PCR analysis to confirm the *JK-1* expression using *GAPDH* expression as control. Approximately $1x10^5$ of both transfected NIH 3T3 and HEK 293 cells were subsequently examined by cell growth assay, soft agar assay as well as foci formation assay, performed as previously described (6). The doubling times of the cells were calculated as reported previously (14).

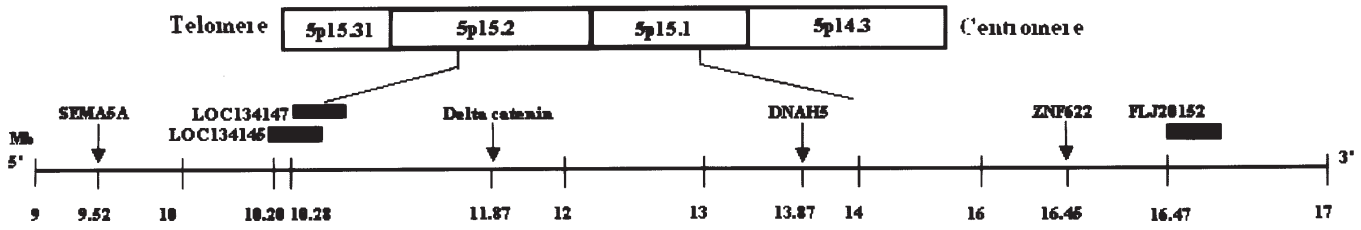

Figure 1. The chromosomal positions of *JK-1* (FLJ20152), *JS-1* (LOC134147) and *JS-2* (LOC134145) genes relative to the δ-catenin gene on chromosome 5p15.1-15.2. *JK-1* is 3' downstream to the δ-catenin gene, while *JS-1* and *JS-2* are 5' upstream to the δ-catenin gene. The figure is not drawn to scale.

ummary of *JK-1* expression studies and the pathological data of esophageal cancer patient cases.

Patient no.	Age/sex	Histopathological type	TNM	Overall stage	*JK-1* expression
1	68/M	SCC (P)	T3N1M0	III	O
2	77/M	SCC (W)	T4N1M0	III	O
3	60/M	SCC (P)	T3N1M0	III	O
4	62/M	SCC (M)	T3N0M0	II	O
5	59/M	SCC (M)	T3N1M0	III	O
6	58/M	SCC (M)	T3N0M0	II	O
7	69/M	SCC (P)	T3N0M0	II	O
8	65/M	SCC (P)	T3N0M0	II	O
9	66/F	SCC (W)	T3N0M0	II	O
10	69/M	SCC (P)	T3N0M1	IV	U
11	66/M	SCC (W)	T3N0M1	IV	U
12	57/M	SCC (M)	T3N0M0	II	U
13	47/M	SCC (M)	T3N1M0	III	U
14	63/M	MEC*	T3N1M0	III	U
15	69/M	SCC (M)	T3N1M0	III	U
16	73/M	SCC (M)	T3N1M0	III	U
17	57/M	SCC (W)	T4N0M0	III	U
18	45/M	SCC (P)	T4N1M0	III	U
19	53/F	SCC (P)	T3N0M0	II	U
20	77/F	SCC (P)	T3N1M0	III	U
21	57/M	SCC (W)	T4N0M0	III	U
22	72/M	SCC (W)	T3N0M0	II	U
23	70/M	SCC (M)	T3N0M0	II	U
24	52/M	SCC (W)	T4N0M0	III	NC
25	76/M	SCC (M)	T3N0M0	II	NC
26	70/M	SCC (M)	T3N0M1	IV	NC
27	59/M	SCC (M)	T3N0M0	II	NC
28	70/M	SCC (W)	T3N1M0	III	X
29	74/M	SCC (M)	T3N1M0	III	X
30	74/M	SCC (M)	T3N0M0	II	X

SCC, squamous cell carcinoma; (W), well-differentiated type of tumor; (M), moderately differentiated type of tumor; (P), poorly differentiated type of tumor; MEC, mucoepidermoid carcinoma; NC, no change in expression; O, overexpression; U, underexpression; X, no expression. *A non-ESCC case.

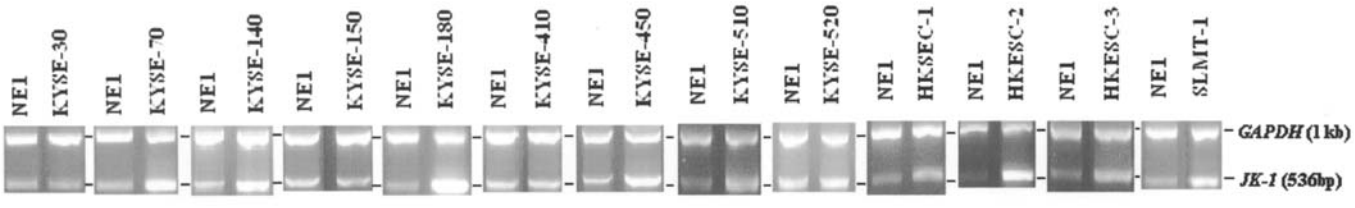

Figure 2. Multiplex RT-PCR analysis for *JK-1* expression in ESCC cell lines. Comparison of expression level of *JK-1* was made between NE1 and ESCC cell lines. NE1 represented a non-tumor epithelial cell line and *GAPDH* expression was used as an internal control. ESCC cell lines of KYSE 70, 140, 180, 450, 510, HKESC-1, HKESC-2, HKESC-3 and SLMT-1 showed overexpression of *JK-1*, while KYSE 30, 150, 410 and 520 showed underexpression.

Tumorigenicity test in nude mice. Approximately 5x10^6 3T3/JK-1 and 3T3/vec cells were subcutaneously injected separately into the flanks of 3 athymic nude mice to assess the tumorigenic potential of JK-1 when it is overexpressed. After one month, the mice were sacrificed and the observable tumors were excised for histopathologic analysis. *JK-1* expression was studied in the parental NIH 3T3, 3T3/vec and 3T3/JK-1 cells by RT-PCR using *GAPDH* expression as control as described in the previous section.

Bioinformatic analysis. Nucleic and amino acid homology searches (http://www.ncbi.nlm.nih.gov/BLAST) were performed with the Basic Local Alignment Search Tool (BLAST) software in the National Center of Biotechnology

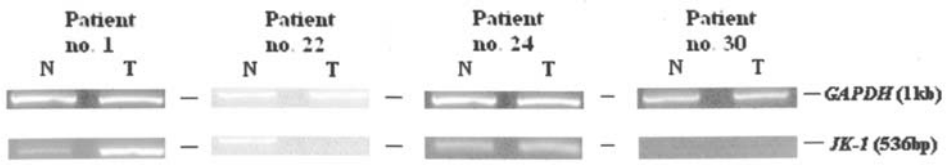

Figure 3. Selected results of the multiplex RT-PCR analysis for *JK-1* expression in ESCC tissue specimens. *GAPDH* expression was used as an internal control. N, non-tumor esophageal epithelium; T, ESCC tissue. The patient numbers are referred to in Table I.

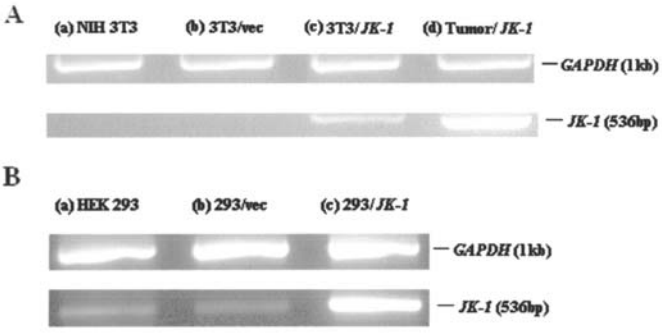

Figure 4. (A) Multiplex RT-PCR analysis for *JK-1* expression in (a) NIH 3T3 cells, (b) 3T3/vec cells, (c) 3T3/*JK-1* cells and (d) subcutaneous tumor tissue from athymic nude mice injected with 3T3/*JK-1* cells (Tumor/*JK-1*). Both 3T3/*JK-1* cells and subcutaneous tumor tissue from athymic nude mice with 3T3/*JK-1* showed overexpression of *JK-1* when compared with parental NIH 3T3 and 3T3/vec cells. (B) Multiplex RT-PCR analysis for *JK-1* expression in (a) HEK 293, (b) 293/vec and (c) 293/*JK-1*. The 293/*JK-1* cells showed overexpression of *JK-1* when compared with parental HEK 293 and 293/vec cells. The *GAPDH* expression served as the internal control.

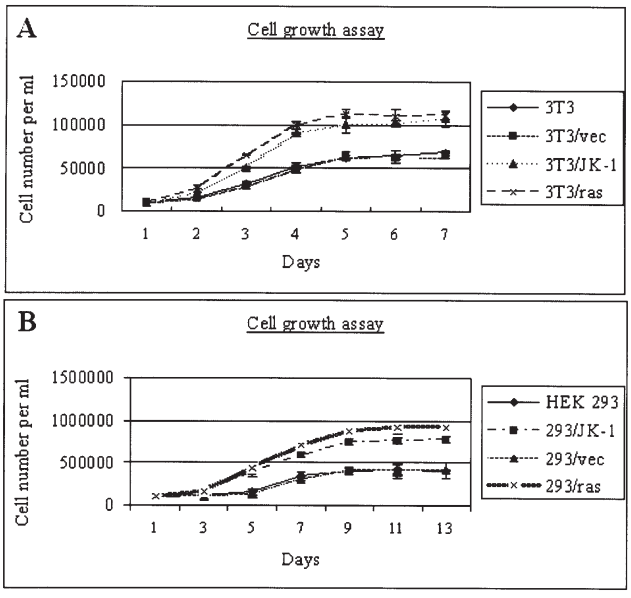

Figure 5. (A) The growth properties of parental NIH 3T3, 3T3/vec, 3T3/*JK-1* and 3T3/*ras* cells. (B) The growth properties of parental HEK 293, 293/vec, 293/*JK-1* and 293/*ras* cells. Cells were grown under conditions as described in Materials and methods. Cells were counted on indicated days with a hemocytometer. All results are the means of triplicate experiments, and the standard deviations are indicated by the standard bars.

Information (NCBI). A ScanProsite search (http://ca.expasy.org) was used to scan the patterns or activation and catalytic sites of the protein sequence of *JK-1*, and MotifScan (http://myhits.isb-sib.ch/cgi-bin/motif_scan) was employed to scan the protein motifs of *JK-1*. GIBRAT (GOR3) secondary structure prediction was carried out to predict the secondary structure. The compute pI/Mw tool (http://ca.expasy.org/tools/pi_tool.html) was used to predict the theoretical pI and molecular weight of *JK-1* protein.

Results

JK-1 expression in ESCC cell lines and tissue specimens. The expression of *JK-1* was significantly elevated by at least 1.5-fold in 69% (9/13) of the ESCC cell lines compared with the immortalized non-tumor esophageal epithelial cells, and about 31% (4/13) of the cell lines showed no change in *JK-1* expression level (Fig. 2). In addition, *JK-1* was overexpressed by at least 1.49-fold in 30% (9/30) of the esophageal tumor tissue specimens compared with their corresponding non-tumor epithelial tissues, and 47% (14/30), 13% (4/30) and 10% (3/30) of them showed underexpression, no change in expression and no expression, respectively (Fig. 3 and Table I). Of the 30 patients tested, 12 were in stage II (40%), 15 were in stage III (50%) and 3 were in stage IV (10%). Concerning the histological features, 27% (8/30) were well-differentiated, 43% (13/30) were moderately differentiated, 27% (8/30) were poorly differentiated and 3% (1/30) was mucoepidermoid carcinoma (Table I). In the present study, 30% (9/30) of the patients overexpressed *JK-1*. *JK-1* overexpression was common in patients with stage II (56%; 5/9) and stage III ESCC (44%, 4/9). In all of the 9 cases overexpressing *JK-1*, 89% (8/9) and 11% (1/9) were of tumor stage T3 and T4 respectively, 55% (5/9) and 45% (4/9) were of stage N0 (without regional lymph node metastases) and N1 (with regional lymph node metastases), respectively. All of the 9 cases overexpressing *JK-1* were at stage M0 (without distant metastasis). Among the 9 ESCC tissue specimens with *JK-1* overexpression, 45% (4/9), 33% (3/9) and 22% (2/9) were poorly, moderately and well-differentiated tumor cells, respectively (Table I).

Cell growth assay. RT-PCR analysis of *JK-1* expression in NIH 3T3, 3T3/vec, 3T3/*JK-1*, HEK 293, 293/vec and 293/JK-1 cells was performed to confirm a successful transfection of *JK-1* into the non-tumor fibroblast cell lines (Fig. 4A and B). The doubling time for NIH 3T3 cells was 25.15 h, while the 3T3/*JK-1* cells required 20.26 h (Fig. 5A). In order to eliminate the action of the vector contributing to a growth advantage in the 3T3 cells, the doubling time of the

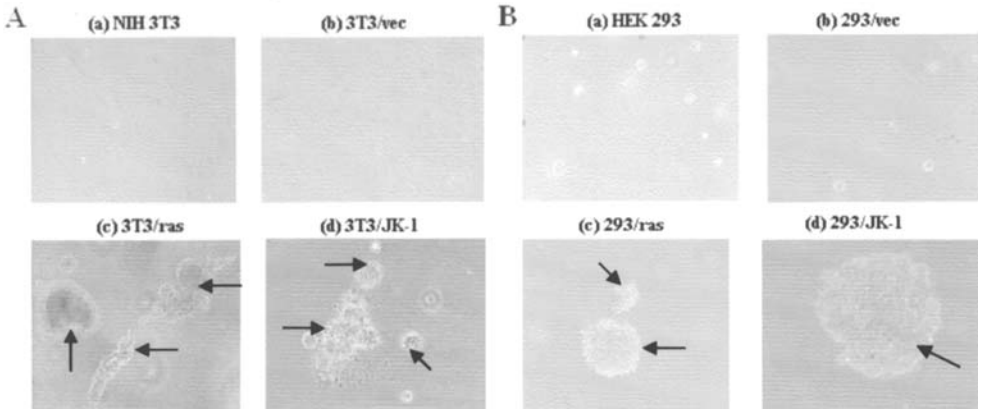

Figure 6. (A) Soft agar assay of (a) NIH 3T3, (b) 3T3/vec, (c) 3T3/*ras* and (d) 3T3/*JK-1*. Colonies (arrows) were formed in NIH 3T3 cells transfected with *JK-1* and pcDNA3.1-H-*ras*V12, but not in those transfected with mock vectors and NIH 3T3 cells. Photos were taken after 3 weeks of cell growth in soft agar. Original magnification, x200. (B) Soft agar assay of (a) HEK 293, (b) 293/vec, (c) 293/*ras* and (d) 293/*JK-1*. Colonies (arrows) were formed in HEK 293 cells transfected with *JK-1* and pcDNA3.1-H-*ras*V12, but not in those transfected with mock vectors and HEK 293 cells. Photos were taken after 4 weeks of cell growth in soft agar. Original magnification, x200.

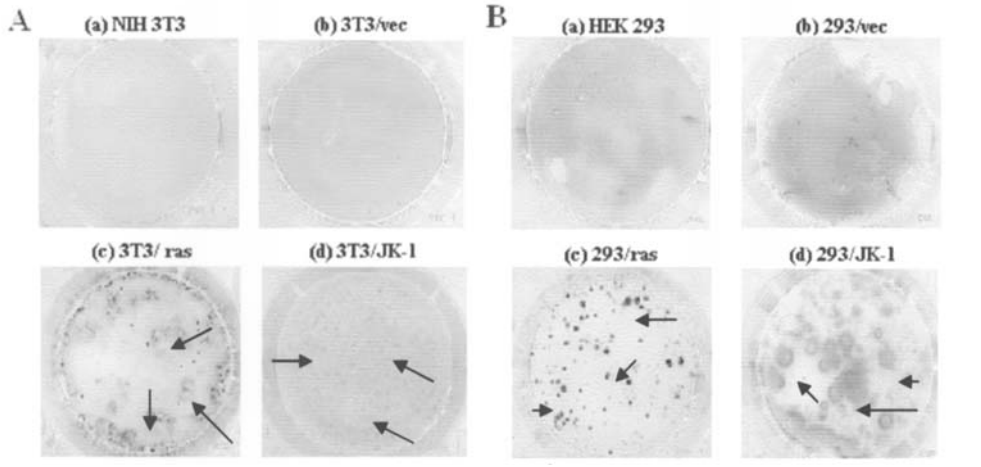

Figure 7. (A) Foci formation assay of (a) NIH 3T3, (b) 3T3/vec, (c) 3T3/*ras* and (d) 3T3/*JK-1*. Foci (arrows) were formed in NIH 3T3 cells transfected with pcDNA3.1-*JK-1* and pcDNA3.1-H-*ras*V12, but not in those transfected with mock vectors and NIH 3T3 cells. Photos were taken after 10 days of seeding cells. (B) Foci formation assay of (a) HEK 293, (b) 293/vec, (c) 293/*ras* and (d) 293/*JK-1*. Photos were taken after 2 weeks of seeding cells. Foci (arrows) were formed in HEK 293 cells transfected with pcDNA3.1-*JK-1* and pcDNA3.1-H-*ras*V12, but not in those transfected with mock vectors and HEK 293 cells.

3T3/vec cells was also estimated at 25.23 h (Fig. 5A). The 3T3 cells with or without empty vector transfection had a similarly observed growth rate. In addition, the results showed that the 3T3/*ras* cells only required 18.16 h to achieve cell doubling (Fig. 5A). Both HEK 293 and 293/vec cells took a longer time for cell multiplication; their doubling times were similar at 41.6 and 41 h respectively (Fig. 5B). Hence, transfection of the vector alone to the HEK 293 cells caused no remarkable change in cell growth rate while the 293/JK-1 and 293/*ras* cells replicated rapidly requiring only 36 and 34.4 h respectively to double their cell numbers (Fig. 5B).

Soft agar assay. The positive controls 3T3/*ras* and 293/*ras* cells formed colonies in soft agar. Similarly, both 3T3/*JK-1* and 293/*JK-1* cells also exhibited an anchorage-independent growth manner, while no colony was observed in the negative controls including parental NIH 3T3, parental HEK 293, 3T3/vec and 293/vec cells (Fig. 6A and B).

Foci formation assay. The 3T3/ras, 293/ras, 3T3/*JK-1* and 293/*JK-1* cells with confluent growth exhibited loss of contact inhibition by foci formation on the tissue culture plates. However, no foci formation was observed on the cells of parental NIH 3T3 and HEK 293 cells as well as 3T3/vec and 293/vec cells (Fig. 7A and B).

Tumorigenicity test in nude mice. The *in vivo* tumorigenicity potential of overexpression of *JK-1* was examined by subcutaneously injecting the 3T3/*JK-1* cells into 3 athymic nude mice. Subcutaneous tumors were formed in all tested mice (3/3) at the site of injection. In contrast, no tumors were observed in the other 3 tested mice injected with the 3T3/vec cells. The tumors were detectable by palpation 1 week after injection. The mice were sacrificed and the tumors were excised for analysis of the histopathologic features at day 43 after injection. Histopathological examination of the *JK-1*-induced tumor cells was performed after collection of the

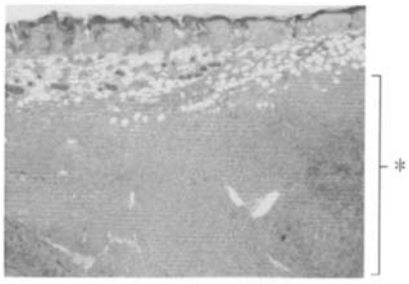

Figure 8. Histological analysis on the subcutaneous tumor formed in the athymic nude mice injected with 3T3/*JK-1* cells showing the morphology of the high-grade sarcoma (*). Hematoxylin and eosin stained; original magnification, x200.

subcutaneous mouse tumors. The tumors induced by 3T3/*JK-1* were hypercellular and composed of spindle tumor cells with prominent nucleoli and frequent mitotic figures indicative of high-grade sarcoma (Fig. 8). The expression level of *JK-1* in the subcutaneous tumors of the tested mice was confirmed by extracting the RNA from the excised tumors, followed by performing RT-PCR analysis, with the parental NIH 3T3 and 3T3/vec cells included as controls. *JK-1* was over-expressed in subcutaneous sarcomas when compared with the controls (Fig. 4A).

Bioinformatic analysis. The results indicated that the coding sequence of *JK-1* indicated a 100% (1071/1071) match with a known human cDNA transcript (accession no. AK024920) in the Genbank database. Based on the results of a protein search, the protein encoded by *JK-1* showed no homology to known functional protein. ScanProsite was used to scan the protein sequence of *JK-1* for the occurrence of patterns stored in the PROSITE database, which displayed that *JK-1* contained an epidermal growth factor (EGF)-like domain signature 1 (1106-1117 bp, CACCTGATGAGC). The molecular weight and pI of the *JK-1* protein were 39.32 kDa and 4.39 respectively as predicted by the compute pI/Mw tool. The MotifScan analysis found that the *JK-1* protein consisted of one protein kinase C phosphorylation site (135-137 SHK), one cAMP and cGMP-dependent kinase phosphorylation site (343-346 KKSS), three N-glycosylation sites (36-39 NFSI, 175-178 NGTF, 179-182 NLSE), three N-myristoylation sites (75-80 GVILSY, 215-220 GMGTND, 290-295 GVQQAL) and ten casein kinase II phosphorylation sites (18-21 SKPD, 128-131 SEAD, 135-138 SHKD, 140-143 SELD, 161-164 SVSD, 171-174 SWTD, 190-193 TSDD, 218-221 TNDE, 306-309 TDTE, 320-323 SELD). The secondary structure prediction indicated that the *JK-1* protein was composed of 171 α helices (48.03%), 57 extended strands (16.01%) and 128 random coils (35.04%).

Discussion

Although chromosome 5p is frequently amplified in different tumor types, potential oncogene candidates residing in this chromosome region and the relationship between 5p gain and patient prognosis have rarely been described in the literature. According to previous studies, the genes located on 5p that may relate to ESCC include the *human telomerase* gene (*hTERT*) (5p15.33) (6), *MLVI2* gene (5p14) (15), *S-phase kinase-associated protein 2* gene (*SKP2*) (5p13) (6), *cyclin A* (16), *prostaglandin E receptor 2* gene (*PTGER2*) (5p13.1-5p13.1) (16), *cadherin 6* (*CDH6*) (5p15.1-p14), *cadherin 12* (*CDH12*) (5p14-p13) and *cadherin 14* (*CDH14*) (5p15.2-p15.1) (17), *uracil-DNA glycosylase 2* gene, and *complement 6* (5p13) and *component 7* genes (5p13) (18). In addition, previous evidence showed that a gain of 5p15 was significantly linked with the survival rate, distant organ metastasis after surgery, and disease recurrence in ESCC patients (19). Our group also recently focused on the identification of genetic target(s) within the 5p amplicon. Our previous studies on ESCC confirmed the tumorigenic and overexpression properties of a novel gene *JS-1* located in chromosome 5p15.2 (6). An overexpression of *JS-1* in NIH 3T3 cells caused an increase in growth rate, colony formation in soft agar and foci formation in confluence growth. Moreover, high-grade sarcoma was also formed in the athymic nude mice when NIH 3T3 cells overexpressing *JS-1* were injected subcutaneously (6).

Our present results showed a noticeable overexpression of *JK-1* in 9/13 (69%) of ESCC cell lines and 9/30 (30%) of ESCC patient cases. In addition, *JK-1* was found over-expressed at least 1.4-fold in both ESCC cell lines and patient tissue specimens. Also, both NE1 and 27/30 (90%) of the non-tumor epithelial tissues from ESCC patients showed *JK-1* expression, suggesting that *JK-1* might play some functional roles in both non-tumor and tumor cells in esophageal squamous cells. Our results also suggested that an over-expression of *JK-1* was not uncommon in the studied cell lines and patient specimens of ESCC. It is possible that the increased expression of *JK-1* as well as its gene product might relate to uncontrolled cell growth and subsequent ESCC development. However, some of our ESCC tissue specimens showed *JK-1* underexpression which might relate to other genetic events, such as chromosome deletion and/or hypermethylation of the *JK-1* promoter (20). Overexpression of genes is relevant in the molecular pathogenesis of ESCC. For example, an overexpression of the nuclear factor *cyclin D1* has been commonly demonstrated in ESCC cell lines as reported by Doki *et al* (21), and tumor samples from ESCC (21) and it may provide growth advantage and enhance tumorigenesis in ESCC (22). Additionally, an overexpression of a signal transducer, *ras*, has been suggested to be related to a higher proliferative state of ESCC cancer cells when compared with their corresponding normal cells (22). According to the discussions in literature (23-25), *bcl-2*, which encodes a molecule with anti-apoptotic effect, has been found overexpressed in 32-74% of ESCC.

Previous studies of CGH showed genomic amplification of chromosome 5p regions in high percentages of ESCC cell lines (1) and patient cases of Hong Kong Chinese origin (2). As mentioned previously, 5p was the 3rd most frequently detected site of DNA gain in primary ESCCs exhibiting chromosomal imbalances (51.9%, 27 cases out of 52 patients) (2). Besides ESCC, overrepresentations of chromosome 5p have been found in other tumors, including breast, colon, head and neck, lung and uterine cervix cancer and oeteosarcoma (26). This observation implied that the 5p region is likely to

ogene(s) which are related to cancer formation, and lification in cancer cells reflects frequently involved oncogenes whose increased expression confers a selective advantage on tumor cell growth (27).

We also observed correlations between overexpression of *JK-1* and the clinico-pathological parameters of the studied ESCC patients, including histopathological type of tumor, tumor stage and histological stage of ESCC. In our study, *JK-1* overexpression was common in patients with stage II (5/9, 56%) and stage III ESCC (4/9, 44%). Also, 89% (8/9) and 11% (1/9) of the cases having tumors overexpressing *JK-1*, at the same time, were reported to have paraesophageal tissue invasion (T3) and adjacent structure invasion (T4), respectively. Also, 44% (4/9) and 56% (5/9) of the tissue specimens showed regional lymph node metastases (N1) and no regional lymph node metastases (N0), respectively. *JK-1* overexpression may be relevant to tumor invasiveness and metastasis; hence, it is necessary to use a larger sample size of ESCC specimens for further examination of whether an overexpression of *JK-1* in ESCC tumor cells could be a potential prognostic marker for determining regional lymph node metastatic potential, invasiveness and aggressiveness of tumors.

As reported previously, the assays for detecting altered growth characteristics *in vitro* of tumor features, including change in growth rate, colony formation in soft agar and contact inhibition growth as foci formation, have been widely used to study the transformed phenotypes since the 1970s (28). We have evaluated the characteristics of the transformed phenotypes in terms of growth rate, contact inhibition and anchorage-independent growth. The results indicated that the non-tumor NIH 3T3 and HEK 293 cells with *JK-1* overexpression acquired the transformed phenotypes, for example rapid growth rate, loss of contact inhibition and anchorage-independent growth in soft agar. Hence, our results showed that the *JK-1*-overexpressed NIH 3T3 and HEK 293 cells behaved similarly to transformed tumor cells, losing their proliferation controls and increasing their cell numbers independent of a physiological need.

The parental cells transfected with the well-characterized *ras* oncogene achieved a much faster growth rate than those transfected with *JK-1*; this implied that both ras and *JK-1* proteins contributed a growth advantage to both NIH 3T3 and HEK 293 cells in terms of growth rate. That the growth patterns and rates for 3T3/vec and 293/vec cells were close to their untransfected parental cells indicated that the cloning vector alone did not give rise to a growth advantage to both NIH 3T3 and HEK 293 cells. However, these non-tumor cells increased their growth rates when *JK-1* was overexpressed. Thus, our results of cell growth rate measurement supported that overexpression of *JK-1* contributed to a selectable growth advantage of normal cells, and this may be relevant to the transformation of normal cells to cancer cells.

Another measure used to determine the altered growth features of transformed cells was soft agar assay, which is a simple quantitative method for assessing the capacity for anchorage-independent growth in a cell line. Soft agar assay for colony formation is the most stringent assay for detecting the malignant transformation of cells (29), and the use of the NIH 3T3 mouse fibroblastic cell line to assay the transforming ability of potential and known oncogenes is well-documented (6). In this study, *JK-1* was transfected and overexpressed in both NIH 3T3 and HEK 293 cells. They both showed a strong anchorage-independent growth manner by forming colonies in soft agar, while no colony was observed in mock vector control and the parental cells. Moreover, normal human stromal cells will not grow on each other when they grow on a tissue culture dish or flask since their proliferation rate will be reduced once they make contact with neighbouring cells, a property known as contact inhibition (30). Conversely, tumor or transformed cells show no reduction in their cell division rate even at high cell densities when cells make contact with each other, and hence, foci (a multilayer pattern of cells) are formed. Both 3T3/*JK-1* and 293/*JK-1* cells caused foci formation once they were confluent to achieve a high yield of cells per unit area, whereas foci were absent in the negative mock control vector and parental cells. Hence, the results of both soft agar and foci formation assays suggested an association between *JK-1* overexpression and the transformation of normal cells to tumor cells in the form of losses in anchorage dependency as well as contact inhibition.

In addition, the tumorigenicity test in nude mice is recognized as the most reliable indicator of malignancy (6). In this test, we demonstrated that a high-grade sarcoma was formed in 3 athymic nude mice with subcutaneous injection of 3T3/*JK-1* cells. Our overall results of transforming and nude mice tumorigenicity assays collectively implies that overexpression of *JK-1* contributes to the malignant transformation of non-tumor NIH 3T3 and HEK 293 cells relevant to tumor formation in ESCC.

The bioinformatic analysis showed that the coding sequence of *JK-1* had a 100% (1071/1071) match with a known human cDNA transcript (accession no. AK024920) in the Genbank database. Hence, we believe that the *JK-1* transcript exists in human cells, and BLAST searching revealed that the *JK-1* protein is a novel protein with an unknown function. In addition, bioinformatic analysis also showed that the *JK-1* protein consisted of many types of domains including some important phosphorylation sites. Therefore, the *JK-1* protein is assumed to be involved in some physiological processes through these domains, and an overexpression of *JK-1* may lead to the disorder of its downstreaming gene(s) resulting in ESCC formation indirectly. This information may become the valuable basis for studying the molecular mechanisms of *JK-1* in the pathogenesis of ESCC.

Gene amplification was regarded as one of the major genomic aberrations and a common mechanism for oncogene overexpression and/or for activating proto-oncogenes (22). It was assumed that the amplified DNA might include some critical genes whose expression provided a selective force for tumor development (4). As a result, regional amplification of 5p might cause extra copies of *JK-1* in the tumor cells, and eventually, an overexpression of *JK-1*. Our results implicated that *JK-1* upregulation might induce or contribute to tumor formation, a rapid growth of tumor cells, unscheduled cell proliferation and malignancy through an interaction of *JK-1* and other candidate gene(s). Hence, our present study showed that an overexpression of *JK-1* might be relevant to

the pathogenesis and progression of ESCC. However, the correlation between *JK-1* expression and ESCC prognosis is not clear. Recently, research related to esophageal squamous cell carcinomas has involved considerable efforts to explore the critical molecular determinants in the development and progression of this disease. The potential candidate genes that have been recently studied include *JK-1* in the present study, *JS-1* (6) and *EC97* (31), and the related candidate tumor suppressor genes *ECRG1* (32) and *TSLC1* (33). Both *JS-1* and *JK-1* were shown to be overexpressed in most ESCC cells. Thus, these two novel genes may likely be the major driving force within the 5p amplicon in ESCC development.

As the 5p is a large region and the functional roles and the mechanism of *JK-1* in the molecular pathogenesis of ESCC are still unknown, further effort is needed to elucidate the significance of the chromosome 5p region in relation to the molecular pathogenesis of ESCC. This may be useful in providing insights into mechanisms of malignant transformation and progression, and identifying critical genes, proteins, and pathways and identifying novel targets for anti-cancer treatments in ESCC and other cancers. In addition, our results hopefully will serve as targets for the investigation of future therapy related to ESCC or other cancers.

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