Junctional adhesion molecule-A, an epithelial–mesenchymal transition inducer, correlates with metastasis and poor prognosis in human nasopharyngeal cancer

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Junctional adhesion molecule-A (JAM-A) is preferentially concentrated at tight junctions and influences epithelial cell morphology and migration. Epithelial-to-mesenchymal transition (EMT) is the conversion process of epithelial cells into mesenchymal cells, and it plays an important role in the invasiveness and metastasis of various cancers. However, the role of JAM-A in regulating the invasive behaviours of human nasopharyngeal carcinoma (NPC) is unknown. In this study, we found that JAM-A upregulation induced EMT, whereas silencing of endogenous JAM-A expression reversed EMT. Furthermore, upregulation of JAM-A led to EMT via activation phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) pathway. PI3K inhibitors blocked JAM-A-induced EMT, suggesting that the kinase acts downstream of JAM-A. Finally, results from 172 human patients with NPC showed that high expression levels of JAM-A correlated with metastasis and poor prognosis in NPC. Taken together, these results suggest that high JAM-A expression positively correlates with poor prognosis in patients with NPC, and induces EMT of NPC cells in vitro and in vivo via the PI3K/Akt pathway. These data indicate novel functions in the JAM-A repertoire, and have clinical implications for the treatment of patients with NPC.

Introduction

Junctional adhesion molecule-A (JAM-A), also known as JAM-1 or F11R, belongs to a family of cell adhesion molecules that localize at intercellular junctions, and mediate several different physiological processes, including intercellular junction assembly and cell polarity (1), cell morphology (2) and leukocyte migration (3). JAM-A is expressed by a number of cell types including epithelial, endothelial cells and dendritic cells; leukocytes and platelets (4,5). In epithelial cells, JAM-A is preferentially concentrated at tight junctions where it is involved in promoting cell-to-cell adhesion. Although JAM-A is expressed at high levels in the enriched hematopoietic stem cell fraction (6), the roles of JAM-A in tumour growth and dissemination are still debated (4,7,8).

Epithelial-to-mesenchymal transition (EMT) is the process of converting epithelial cells into mesenchymal cells, and is a central mechanism responsible for the invasiveness and metastasis of various cancers (9,10). EMT is defined by loss of the epithelial phenotype and the acquisition of mesenchymal characteristics, such as migratory capacity, polarity reversal and loss of cell-to-cell contacts. One of the earliest steps in EMT is the loss of E-cadherin function (11). Several transcription factors are reported to drive EMT, including members of the Snail family, zinc finger E box-binding homeobox (ZEB) and the basic helix-loop-helix transcription factor Twist (12). Moreover, it has been reported that the phosphatidylinositol-3 kinase (PI3K) downstream effector, protein Kinase B (PKB, also known as Akt), is indispensable for the upregulation of Twist and Slug in squamous cell carcinoma and liver cancer (13,14). Together, these diverse lines of evidence suggest a possible link between JAM-A and EMT. Thus, we sought to identify the role of JAM-A in regulating the invasive behaviour of human nasopharyngeal carcinoma (NPC), which is a fairly rare cancer in Western Europe and North America, but has a higher incidence in Southern China. Our results indicate that JAM-A upregulation induces EMT of NPC cells in vitro, whereas silencing of endogenous JAM-A reversed EMT. Furthermore, upregulation of JAM-A led to EMT via activation PI3K/Akt pathway. Treatment with PI3K inhibitors blocked JAM-A-induced EMT, suggesting that the kinase acts downstream of JAM-A. Finally, we assessed 172 human patients with NPC and found that high JAM-A expression correlated with metastasis and poor prognosis in NPC. Taken together, these results suggest that high expression levels of JAM-A positively correlate with poor prognosis in patients with NPC, and induce EMT of NPCs via the PI3K/Akt pathway. These data indicate novel functions in the JAM-A repertoire and have clinical implications for the treatment of patients with NPC.

Materials and methods

Cell culture and reagents

CNE2 and HONE1 cell lines were available at the Cancer Institute of Southern Medical University (Guangzhou, Guangdong province, China) and were originally purchased from the American Type Culture Collection (ATCC, Manassas, VA). They have been confirmed negative for mycoplasma in 2012. The authenticity of cell lines in our study have verified with the DNA fingerprinting method in 2012 (15). This laboratory has never grown HeLa cells. Both cell lines were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) and antibiotics. LY294002 was obtained from Calbiochem (San Diego, CA).

Lentiviral vectors construction

The stably overexpressing JAM-A cell lines CNE2-pWPI-JAM-A and HONE1-pWPI-JAM-A and their respective control cell lines were transduced with either pWPI encoding human JAM-A or empty pWPI vector, respectively. The pWPI lentiviral vector system was obtained from Addgene (Cambridge, MA). The human JAM-A gene (NM_016946.4) was cloned from CNE2 cells by polymerase chain reaction (PCR) using the primers shown in Supplementary Table S1, available at Carcinogenesis online. Gene identification was confirmed by agarose gel electrophoresis, and then a JAM-A fragment generated digestion with PaeI and PmeI restriction enzymes were ligated into a linearized pWPI plasmid by digestion with the same enzymes. The resulting recombinant vector was transformed into competent Escherichia coli DH5st cells to construct a JAM-A overexpressing vector. Finally, the recombinant vector was confirmed by sequencing.

For JAM-A short hairpin RNA (shRNA) constructs, the pRS (retro-super)-constructs containing JAM-A shRNA were generated by cloning two JAM-A-specific RNAi target sequences (Supplementary Table S1, available at Carcinogenesis online) into pRS. Transient transfection of cells with scramble RNAs or siRNAs targeting JAM-A mRNA was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), in accordance with the manufacturer’s protocol. The effects of shRNAs were confirmed by three independent transfection experiments.

Lentiviral packaging and lentiviral infection

The recombinant lentiviral vector, packing mix (Invitrogen) and Lipofectamine were mixed according protocol of Lipofectamine. The resulting mixture was...
added to the 293T cells, which were maintained in Opti-MEM and incubated in 5% CO₂ at 37°C for 6 h. After refreshing the culture medium with complete medium [ Dulbecco’s Modified Eagle’s medium (DMEM) + 10% FBS], the cells were incubated for 72 h and the supernatant was collected, followed by centrifugation at 1300 g for 8 min and filtering through a 0.45 μm filter. Finally, the supernatant was centrifuged at 60,000 g for 2 h and the precipitate was collected. The virus was resuspended in DMEM and stored at −80°C for further use. When cell confluence reached 80%, the medium was removed and the cells were washed with phosphate-buffered saline (PBS). After cells were incubated for 16 h, the supernatant was removed, and the cells were washed and maintained in complete medium. The infection efficiency was evaluated by counting cells in five high-power fields under a microscope (Nikon, Tokyo, Japan).

**Migration and invasion assays**

These assays were performed as described previously (16). Briefly, for transwell and Boyden chamber assays, cells with or without regulation JAM-A were serum-starved for 24 h, then 2 × 10⁵ cells were plated into the upper chamber and incubated for 22 h. For wound-healing assay, the cells reaching 90% confluence were then serum-starved for 24 h, and similar sized wounds were made by scraping a conventional 10 μl micropipette tip across the monolayer.

**Anchorage-independent growth assay**

To measure anchorage-independent growth, cells were detached with trypsin and resuspended in growth medium. Six well-plates were coated with 0.75% agarose (Sigma–Aldrich, St Louis, MO) in growth medium to form base layer and then overlaid with a suspension of cells in 0.45% agarose. Plates were incubated for 3 weeks at 37°C and colonies were counted under microscope.

**Gelatin zymography assay**

The gelatinolytic activity of matrix metalloproteinases-2 (MMP-2) and MMP-9 was determined by gelatin zymography. Conditioned medium was collected after 24 h and diluted in sample buffer (50 mMol/l Tris (pH 6.8), 0.5% SDS, 10% glycerol, 0.2% bronopol blue). Then, they were separated by electrophoresis on a 7.5% polyacrylamide gel containing 1 mg/ml gelatin as a substrate. Gels were washed with 2.5% Triton X-100 (Sigma–Aldrich), followed by overnight incubation in reaction buffer. Finally, gel was stained with a 5% Coomassie solution (Bio-Rad, Hercules, CA).

**Immunofluorescence analysis**

Cells that were 70–90% confluent were stained for immunofluorescence on coverslips as described previously (16). Briefly, the cells were incubated with primary antibodies against E-cadherin, fibronectin, JAM-A and vimentin and then incubated with FITC or TRITC-conjugated goat antibodies against rabbit or mouse IgG. The cell nuclei were stained with 4′,6-diamino-2-phenylindole (DAPI) (Invitrogen). The images were recorded by fluorescence microscopy with a Nikon Eclipse 80i microscope (Nikon). The primary antibodies were omitted for negative control staining.

**Mouse xenograft and experimental metastasis assay**

Female BALB/c nude mice (4–6 weeks old) were purchased from the Model Animal Research Center of Nanjing University (Nanjing, Jiangsu province, China). All mouse experiments were approved by the animal care and use committee of Southern Medical University (Guangzhou). To evaluate the effect of JAM-A on tumour growth, cell suspensions (5 × 10⁶ cells/100 μl) were subcutaneously injected into the animals’ right hind limbs. The tumour volume was measured every 3 days for 30 days, and mice were euthanized on day 30. For the experimental metastasis assay, cells stably transfected with scramble or JAM-A-shRNA (2 × 10⁵ cells in 0.2 ml PBS) were injected intravenously into the lateral tail vein of 4-week-old nude mice, and lung colonization was evaluated 6 weeks later. Each treatment group consisted of seven mice. At termination, the lungs were removed. Surface metastatic nodules per lung were counted by overnight incubation in reaction buffer. Finally, gel was stained with a 5% Coomassie solution (Bio-Rad, Hercules, CA).

**Radiation sensitivity assay**

Cells were harvested 24 h after X-ray irradiation at a dose of 4 Gy. Apoptosis assays, including terminal deoxyadenosine deoxynucleotidyl transferase (TUNEL), caspase-3, flow cytometry analysis and western blotting were performed as described previously (16). Cell proliferation was measured by 5-ethyl-2′-deoxyuridine (EdU) assay using an EdU assay kit (RiboBio, Guangzhou, China) according to the manufacturer’s protocol. For colony formation assay, cell suspensions were transferred into six well-plates (300 cells per well) and incubated for 14 days. Surviving cells were fixed in 30% ethanol and stained with 0.25% methylene blue. Colonies containing more than 50 cells were counted, and colony formation rate was calculated as (number of colonies/number of cells) × 100%.

**Western blotting analysis**

In order to reduce the side-effect of different confluency, our samples for western blotting experiments were collected in adherent cultures when cells were 80–90% confluent. Then, protein from cells or tumour tissues were mixed with loading buffer and heated at 70°C for 10 min. They (30 μg/ lane) were separated on SDS-PAGE gels and transferred to polyvinylidene fluoride (PVDF) Millipore, MA). Membranes were blocked for 2 hr in 5% bovine serum albumin (BSA) and incubated overnight at 4°C with the SP rabbit polyclonal antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were then incubated with horseradish peroxidase (HRP)- conjugated secondary antibody (1:1000, Santa Cruz Biotechnology) for 1 h at room temperature. Finally, bands were visualized by enhanced chemiluminescence (ECL, Thermo Scientific Pierce, Rockford, IL).

**Tumour sample immunohistochemistry**

A total of 195 patients with NPC were recruited between January 2005 and December 2007 at the Sun Yat-sen University Cancer Center (Guangzhou, China) and the Armed Police Hospital (Guangzhou, China). For the use of these clinical materials for research purposes, prior written informed consent from all the patients and approval from the Ethics Committees of the Sun Yat-sen University Cancer Center or the Armed Police Hospital were obtained. Histological classification and clinicopathological staging of the samples was performed according to the rules of WHO histological classification. Of these, 172 patients were analysed; the other 23 cases were excluded from this study because the samples were inadequate for staining. The 172 patients included 121 males and 51 females with ages ranging from 31 to 74 years (median age 41 years). All patients were treated with standard curative radiotherapy with or without chemotherapy. JAM-A was detected using rabbit polyclonal antibody (Abcam, Cambridge, UK). For clinical samples, all slides were evaluated independently by two investigators who were blinded to the patients’ clinical data, using a previously described method (17). Negative and positive controls were prepared. We classified the tumours into two major groups based on JAM-A expression: JAM-A-negative (tumours where JAM-A was absent) and JAM-A-positive (tumours with various degrees of JAM-A expression).

**Statistical analysis**

Statistical analysis was performed with SPSS statistical package (v15.0). In vitro experiments were repeated three times and data are presented as the mean ± SD. Statistical differences among groups were assessed with one-way analysis of variance (ANOVA). The chi-square test and Fisher’s exact test were used to analyze the JAM-A expression with other clinicopathological parameters. The chi-square test was also used to analyse the diameters of mammoplasties. Kaplan–Meier analysis and the log-rank test were used to illustrate differences between distant metastasis-free survival (DMFS) and overall survival (OS) rate according to JAM-A expression. Significant variables were further analysed by multivariate analysis to test for independent prognosis. The P-values of less than 0.05 considered statistically significant.

**Results**

**Ectopic expression of JAM-A induces EMT of NPCs in vitro**

Firstly, we sought to study the functional relevance of JAM-A in EMT of NPC cells. Results indicated that a dramatic morphological change was observed in the pWPI-JAM-A/NPC cell lines; they lost their typical cobblestone-like appearance and displayed a spindle-like, fibroblastic morphology (Figure 1A), which suggested that the pWPI-JAM-A/NPC cells might have undergone EMT. Consistently, results of immunofluorescent staining indicated that JAM-A protein levels in cell membrane increased significantly in the JAM-A-overexpressing NPC cells. Moreover, they exhibited the typical EMT phenotype, downregulation of epithelial markers E-cadherin and upregulation of mesenchymal markers fibronectin and vimentin (Figure 1B). Furthermore, we found that JAM-A overexpression led to an obvious enhancement of invasion and metastasis of NPC cell lines (Figure 1C and 1D). As shown in Figure 1E, the migration index of pWPI-JAM-A NPC cells measured by wound-healing assays also increased compared with vector control cells. Next, we utilized a standard zymogen gelatinase assay to determine whether the elevated levels...
Correlation of JAM-A with metastasis and poor prognosis

of JAM-A in NPC cells would be sufficient to increase MMP-2 and MMP-9 activity, both of which play an important role in cancer invasion and metastasis. Interestingly, MMP-2 and MMP-9 activity increased in pWPI-JAM-A NPC cells relative to control cells (Figure 1F). Moreover, western blotting analysis revealed a decrease in the expression levels of α-catenin and E-cadherin, and an increase in the expression levels of the mesenchymal cell markers fibronectin and vimentin in JAM-A-overexpressing NPC cell lines (Figure 1G). Taken together, our results suggest that upregulation of JAM-A is sufficient to induce EMT and enhance invasiveness of NPC cells in vitro.

Downregulation of JAM-A represses the EMT phenotype of NPC cells

To further investigate the effect of JAM-A on EMT, endogenous JAM-A was downregulated in cells using specific shRNAs. Both shRNAs specifically knocked down endogenous JAM-A protein in CNE2 and HONE1 cell lines (Figure 2A). As anticipated, increased E-cadherin expression and reduction of vimentin were detected in both JAM-A down-regulated cells, indicating that knockdown of JAM-A induces mesenchymal-epithelial transition (MET) in NPC cells. However, shRNA #2 was more efficient, and was therefore chosen for subsequent studies. Migration and invasion assays revealed that.
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ablation of endogenous JAM-A markedly reduced NPC cell migration and invasiveness (Figure 2B, 2C and 2D). Consistently, results of immunofluorescent staining indicated that knockdown of JAM-A by siRNA dramatically reduced JAM-A localized in the cell membrane, mesenchymal markers fibronectin and vimentin (Supplementary Figure S1, available at Carcinogenesis online). Moreover, MMP-2 and MMP-9 activity decreased in JAM-A-shRNA cells relative to control cells (Figure 2E). We next examined the effect of JAM-A on the tumorigenic activity of NPC cells using an anchorage-independent growth assay. A strong decrease in anchorage-independent growth was observed (Figure 2F). Altogether, these data indicated that down-regulation of JAM-A represses the EMT phenotype.

We also assessed the in vivo tumorigenicity of JAM-A in a subcutaneous xenograft nude mouse model. At day 30 post-injection, JAM-A-shRNA cells exhibited greatly reduced tumour growth in contrast to scramble cells (Figure 2G and 2H). Moreover, increased E-cadherin expression and decreased vimentin expression in xenograft tumours were observed in the JAM-AsiRNA group as assessed by western blotting (Figure 2I) and immunohistochemistry (Supplementary Figure S2, available at Carcinogenesis online). Interestingly, spindle-like cells were observed in the invasive front of subcutaneous xenografts in some of the control and scramble group. However, vimentin expressed in the invasive front was not different from that expressed in the center of subcutaneous xenografts. Furthermore, the possible effect of JAM-A on metastatic potential in vivo were tested using an experimental metastasis assay. Control scramble cells formed 17.3 (14 to 21) metastatic nodules per lung in all 7 mice analysed, which were evaluated under a dissection microscope. In contrast, 7 nude mice injected with JAM-A shRNA cells formed 8.6 (3 to 10) nodules per lung. H&E staining confirmed that both the number and the volume of micrometastatic lesions were markedly reduced in the lungs of mice injected with shRNA #2 (Figure 2J). Consistently, the results from intrasplenic injection indicated that after 6 weeks, only 5/10 mice injected with JAM-A-shRNA cells presented liver metastases, whereas 8/10 mice injected with JAM-A-scramble cells developed metastases (Figure S3). Taken together, these results suggest that JAM-A is necessary for the aggressive metastatic phenotype of NPC cells.
Correlation of JAM-A with metastasis and poor prognosis

JAM-A mediates NPC cell resistance to radiotherapy

Previous studies have demonstrated that EMT provides a mechanism of cell escape to a new, less adverse niche, and resistance to apoptosis and radiotherapy or chemotherapy (18). TUNEL assay results showed that overexpressing JAM-A cells exhibited decreased apoptotic features 24 h after irradiation, such as DNA fragmentation and nuclear condensation. The percentage of TUNEL positive cells decreased from 15.7% (CEN2-pWPI-vector) to 7.2% (CNE2-pWPI-JAM-A). Interestingly, this decrease in apoptosis could be reversed by JAM-A-shRNA (Figure 3A). Furthermore, decreased caspase-3 activity could be reversed by JAM-A-shRNA (Figure 3B). In addition, the modes of cell death were assessed by flow cytometry. The results were similar, showing that radiation-induced apoptotic cell death was markedly inhibited by JAM-A, and JAM-A downregulation induced a higher percentage of apoptosis (Figure 3C). Finally, western blotting was employed to confirm radiation-induced apoptosis in CNE2 cells. As shown in Figure 3D, JAM-A inhibited poly ADP ribose polymerase (PARP) and Caspase-3 cleavage could be induced by JAM-A-shRNA, indicating that JAM-A inhibits caspase-mediated apoptosis.

We further explored proliferation and colony-formation ability after irradiation in cells with different levels of JAM-A expression. Compared with the JAM-A scramble control cells 24 h after irradiation, the proliferation of JAM-A-overexpressing cells was upregulated by about 30%, but this upregulation could be reversed by JAM-A-shRNA (Figure 3E). Colony formation assays (Figure 3F) also showed that JAM-A-shRNA increased sensitivity to radiotherapy.

Taken together, these results reveal that JAM-A is critical for the regulation of therapeutic sensitivity of NPC cells.

JAM-A induces EMT of NPC cells by activating Akt signalling

To understand whether the mechanisms behind JAM-A-induced EMT are attributable to Akt or extracellular regulated protein kinases1/2 (ERK1/2), the protein levels of phosphorylated Akt (p-Akt) and phosphorylated ERK1/2 (p-ERK1/2) were measured by western blotting. Upregulation of JAM-A did indeed facilitate p-Akt in both CNE2 and HONE1 cells, whereas p-Akt was inhibited in cells transfected with JAM-A-shRNA (Figure 4A and B). However, JAM-A overexpression did not affect the ERK1/2 pathway. To date, although many transcription factors can trigger EMT, the full molecular reprogramming occurring during an EMT is mainly orchestrated by three major groups of transcription factors: the ZEB, Snail and Twist families (12). Thus, our studies investigated the levels of ZEB, Snail and Twist. Interestingly, Slug and Twist, which have been shown to be vital for EMT, increased along with JAM-A and p-Akt, while with no difference in ZEB1 and ZEB2 (data not shown). Moreover, the increase in p-Akt was accompanied by a change in the phosphorylation of glycogen synthase kinase 3 beta (p-GSK3β) and p70 S6 kinase (p70S6K), both of which are downstream effectors of PI3K, suggesting that JAM-A-induced EMT may activate the PI3K/Akt pathway in NPC cells.

NPC cells were also treated with a PI3K inhibitor, LY294002 (10 μM). Results indicated that JAM-A-induced migration and invasion were blocked by LY294002 (Figure 4C). Western blotting revealed that, compared with vector cells, the protein levels of...
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Slug, Twist and vimentin were significantly increased in the pWPI-JAM-A/NPC cell lines but decreased in cells treated with LY294002 (Figure 4D). Moreover, the results of real-time reverse transcriptase (RT)-PCR indicated that the effect of JAM-A on Slug, Twist, and epithelium-related proteins was examined by western blotting. Slug (E), Twist (F), E-cadherin and vimentin (G) expression levels were examined by real-time RT-PCR. Data are presented as mean ± SD (n = 3). Bars with different characters are statistically different at the P < 0.05 level. (H) Proposed signalling pathway by which JAM-A induces EMT in NPC cells. JAM-A acts on the kinase Akt, thereby promoting Slug and Twist nuclear translocation and decreasing the activity of transcription factor for E-cadherin.

High JAM-A expression correlates with metastasis and poor prognosis in NPC

To determine the clinical significance of JAM-A expression in patients with cancer, we performed immunohistochemical labelling of samples from a cohort of 172 human patients with NPC. In JAM-A-positive tissue, positive staining was observed in the tumour cell membrane (Figure 5A). Overall, JAM-A expression was observed in 78 out of 172 patients and was significantly associated with clinical T stage classification (P < 0.05). No correlation was observed between JAM-A expression and age, gender, histological type, N stage or TNM stage (P > 0.05) (Supplementary Table S2, available at Carcinogenesis online).

For all patients, the cumulative 5 year OS rate was 47.7%. Kaplan–Meier analysis and log-rank testing were used to relate JAM-A expression to OS and DMFS. High JAM-A protein expression correlated strongly with poorer OS in patients with NPC (Figure 5B, P < 0.01). In addition, patients whose tumours had positive JAM-A protein expression were significantly more likely to develop a recurrence within 5 years compared with patients with negative JAM-A expression (Figure 5C; P < 0.01). Using univariate analysis of the entire group of patients, N classification (HR = 2.136, P = 0.009), M classification (HR = 5.265, P < 0.01), advanced T classification (HR = 1.746, P = 0.024), clinical stage (HR = 4.252, P = 0.003) and JAM-A overexpression (HR = 3.379, P < 0.01) were significantly associated with poor OS. However, only M classification (HR = 3.986, P = 0.016), clinical stage (HR = 3.248, P = 0.005) and JAM-A overexpression (HR = 4.315, P = 0.002) were independent predictors for OS in multivariate analysis (Table I).

Discussion

JAM-A is preferentially concentrated at tight junctions and influences epithelial cell morphology and migration (1). EMT plays an important role in the invasiveness and metastasis of various cancers (11). Thus, we sought to identify the role of JAM-A in regulating the invasive behaviour of NPC. The results of the present study show that JAM-A induces EMT in NPC cells by activating the PI3K/Akt pathway.
Correlation of JAM-A with metastasis and poor prognosis

Moreover, high expression levels of JAM-A correlates with metastasis and poor prognosis in patients with NPC.

Naik et al. (19) indicated that JAM-A serves as a key negative regulator of breast cancer cell invasion and possibly metastasis. However, our results indicated that JAM-A overexpression increased EMT in an NPC cell line. Consistent with our studies, Mc Sherry et al. (7) demonstrated that JAM-A can drive breast cancer cell migration. Severson et al. (8) confirmed that JAM-A dimerisation facilitates formation of a complex to enhance cell migration. Several other studies have also indicated that JAM-A decreases cell apoptosis and increases cancer progression (4,20). Why does JAM-A, a cell-adhesion protein predominantly expressed at the tight junctions of both endothelial and epithelial cells, enhance tumour cell EMT? This can explained as follows. Firstly, JAM-A is comprised of an extracellular domain with two Ig-like loops and a short cytoplasmic tail. The extracellular domain of JAM-A is known to be involved with tight junctions, and the JAM-A cytoplasmic domain is involved in the recruitment and activation of other molecules (21). The role of JAM-A studied by us may be confined to cytoplasmic domain. Secondly, previous studies have demonstrated a key role of JAM-A in tight junction assembly and epithelial barrier function in a non-tumour intestinal epithelial cell line and in hematopoietic stem cells (6). However, JAM-A has also been implicated in enhancing tumour invasiveness, such as in breast cancer. That is, JAM-A is involved in tight junctions in normal non-tumour cell lines, but it is involved in invasiveness in tumours. Along these lines, it was therefore presumed in our study that JAM-A overexpression would enhance NPC cell EMT. The present study also surveyed the mechanism of action of JAM-A in regulating EMT. Mandell et al. (2) confirmed that JAM-A regulates epithelial cell morphology by modulating activity of the small Ras-proximate-1 (Rap1) GTPase. McSherry et al. (7) provided compelling evidence that JAM-A drives breast cancer cell migration by activation of Rap1 GTPase. Moreover, studies have indicated that the Akt signalling pathway can be activated by Rap1 GTPase (22). It has also been reported that the PI3K downstream effector, Akt, was indispensable for the upregulation of Slug and Twist (13,14). So we hypothesized that EMT induction by JAM-A in NPC cells occurred via the PI3K/Akt pathway. As anticipated, our results indicated that PI3K/Akt was activated by JAM-A. Importantly, addition of PI3K inhibitor resulted in the reversal of JAM-A-induced EMT. It is important to note that this observation does not exclude the possibility that other signalling pathways not investigated here might also affect JAM-A-induced EMT. Nevertheless, these results indicate that JAM-A induces EMT at least in part via the PI3K/Akt pathway.

To our knowledge, this is the first study to examine outcome in relation to JAM-A expression levels in patients with NPC. We found that high JAM-A protein expression levels were associated with significantly poorer OS and poorer DMFS. Mc Sherry et al. (4) and Murakami et al. (7) observed similar correlations in their groups of patients with invasive breast cancer (n = 270 and n = 444, respectively), while Naik et al. (19) found the opposite correlation between JAM-A expression...
and tumour invasion, but they had fewer than 50 samples. Taking into consideration the larger dataset of the first two studies and of the present work we feel rather confident that in the majority of cases, high JAM-A expression is a prognostic factor for poor patient outcome. Additionally, according to Ou Yang et al. (23) the 5 year OS rate for patients with NPC was 82.2%, and the 5 year DMFS was 87.0%. However, in our studies, the 5 year OS and 5 year DMFS rates were 47.7% and 60.3%, respectively. There were some differences in the results between the current and previous studies, possibly because patients with locally advanced NPC (stages III and IV) accounted for about 80% of the patients in our study, compared to ~65% in previous reports.

In conclusion, our data demonstrate for the first time that the tight junction protein JAM-A induces EMT of NPC in vitro and in vivo via the PI3K/Akt pathway. We found that high expression levels of JAM-A positively correlated with poor prognosis in patients with NPC. These data indicate novel functions in the JAM-A repertoire and provide a mechanistic insight into previous findings. Although many aspects remain to be investigated in future research, it is tempting to speculate that biological antibody therapies for JAM-A may be a promising future target for patients with JAM-A-overexpressing NPC.

### Supplementary material

Supplementary Tables S1–S2 and Figures S1–S2 can be found at [http://carcin.oxfordjournals.org/](http://carcin.oxfordjournals.org/)

### Funding

National Natural Science Foundation of China-Guangdong Joint Fund (K1060006), National Science and Technology support program, National Natural Science Foundation of China and Program for New Century Excellent Talents in University.

### Conflict of Interest Statement

None declared.

### References


Received February 13, 2014; revised September 25, 2014; accepted October 24, 2014.

### Table I. Summary of univariate and multivariate analysis of overall survival duration in all NPCs

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