Original Article

Activation of c-Jun/JNK signaling predicts poor prognosis in nasopharyngeal carcinoma

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Abstract: A significant proportion of patients with nasopharyngeal carcinoma (NPC) will develop regional relapse or distant metastasis after treatment. This present study evaluated the role of c-Jun/JNK signaling pathways in NPC and its relationship with prognosis. Our study enrolled 122 patients diagnosed with NPC and 136 chronic nasosinusitis patients. Immunohistochemistry was applied to detect positive expression of c-Jun, JNK, p-c-Jun, and p-JNK proteins. Receiver operating characteristic (ROC) curve was then adopted to assess the diagnostic value of c-Jun/JNK signaling pathways for NPC. Activated c-Jun/JNK signaling pathways were observed in NPC patients. Activation of c-Jun/JNK signaling was associated with TNM staging of NPC, as NPC patients with stage III-IV had higher positive expression rates of c-Jun, JNK, p-c-Jun, and p-JNK proteins compared to NPC patients with stage I-II. According to ROC curve results, areas under the curve of c-Jun, JNK, p-c-Jun, and p-JNK protein expression were 0.943, 0.968, 0.963, and 0.938, respectively. The 1-, 3-, and 5-year survival rates and mean survival times of dead patients were lower and shorter in patients with positive expressions of c-Jun, JNK, p-c-Jun, and p-JNK than those with negative expression (all \( P < 0.05 \)). Overexpression of c-Jun/JNK is associated with development and progression of NPC, indicating that c-Jun/JNK serves as a predictive indicator for early diagnosis and prognosis of NPC.

Keywords: Nasopharyngeal carcinoma, c-Jun/JNK signaling pathways, prognosis, recurrence, diagnosis, c-Jun, JNK

Introduction

Nasopharyngeal carcinoma (NPC) is a head-and-neck malignant epithelial carcinoma with an incidence of less than 1 per 100,000 each year [1, 2]. As reported, there are an estimated 86,500 new cases of NPC, accounting for only 0.6% of all cancers, newly diagnosed in 2012 worldwide [3]. Epstein-Barr (EB), a carcinogenic herpes virus, has been shown to be a major risk factor for NPC and nodal involvement or distal metastasis is commonly observed in initial diagnosis of most NPC patients [4, 5]. Although a better prognosis of this tumor is achieved in an early stage, prognosis worsens in advanced clinical staging [6]. Recently, radiotherapy and chemotherapy have mainly been performed for clinical treatment of NPC, resulting in serious adversity of effects and bad clinical compliance [7]. Interestingly, recent years have witnessed an achievement made in assessment of NPC clinical stage and prognosis at molecular levels [8].

C-Jun NH 2-terminal kinases (JNKs) are members of mitogen-activated protein kinases subfamily and have been linked to the transduction of a wide variety of intracellular signals [9]. Indeed, there have been an increasing number of studies reporting aberrant activation of JNK pathways in a variety of human cancers and the significance of JNK in controlling fundamental cellular features of cancer cells including proliferation, migration, differentiation, development, the inflammatory response, and apoptosis [10, 11]. To the best of our knowledge, JNK activation by oncogenes is increased in some tumor types and JNK signaling can affect certain forms of intestinal cancer due to its protumorigenic function [12]. Interestingly, activation of JNK has been closely related to some human diseases including colorectal and breast
and ovarian cancers, indicating the critical role of JNK in disease pathology [13]. Prolonged activation of JNK leads to inactivation of tumor suppressor protein p53, further contributing to development and progression of NPC and oral squamous cell carcinoma [14]. Thus, based on previous studies, our study aimed to elucidate the effect of c-Jun/JNK signaling pathways on clinical features and prognosis of NPC to provide a novel approach for NPC diagnosis and therapy.

Materials and methods

Study subjects

A total of 122 patients, diagnosed with NPC in Tianjin Medical University General Hospital from January 2009 to December 2011, were recruited as our case group. Patients in the case group were clinically staged based on NPC tumor node metastasis (TNM) staging criteria (7th version) of the Union for International Cancer Control (UICC). Inclusion criteria: (1) Patients diagnosed with NPC by clinical manifestation and biopsy; (2) Patients that had first onset of disease; and (3) Patients that did not receive chemotherapy or radiotherapy before recruitment. Exclusion criteria: patients with serious heart, liver, and kidney dysfunction, immune system, or endocrine system diseases. Patients in the case group underwent classic radiotherapy combined with necessary chemotherapy and the therapeutic regimen was conducted in accordance with treatment guidelines (2008) of NPC by the National Comprehensive Cancer Network (NCCN). Patients, free from serious systematic disease and history of cancer totaling 136, were diagnosed with chronic nasosinusitis and recruited as our benign control group. All subjects underwent fine-needle aspiration to collect a cell mass of nasopharynx tissue for immunohistochemistry staining. All experimental processes were approved by the Ethics Committee of Tianjin Medical University General Hospital and signed informed consent was obtained from all subjects.

Immunohistochemistry staining

EnVision two-step method: fresh NPC samples were obtained, fixed by 4% formaldehyde, embedded in paraffin, water-de-waxed, treated with 3% H₂O₂ for 1 hour to block the activity of endogenous peroxidase (EGPO), and then washed by phosphate buffered solution (PBS, containing 1.9 mM monopotassium phosphate, 8.1 mM dipotassium phosphate, 75 mM sodium chloride, pH 7.4) 3 times (2 min/time). Then, drops of rabbit-anti-human c-Jun (1:250, product’s ID: ab32137), rabbit-anti-human c-Jun N-terminal kinase (JNK) (1:500, product’s ID: ab112501), mouse-anti-human Phospho-Ser63 c-Jun (p-c-Jun) (1:50, product’s ID: ab195924), and Phospho-Thr183/185 JNK (p-JNK) (1:50, product’s ID: ab131499) with monoclonal antibody (Abcam, Cambridge, UK) were added for incubation at 37°C for 1 hour. Samples were taken out and washed with PBS 3 times (2 min/time), after which a general second antibody (EnVision) (ZSGB-Bio Ltd., Beijing, China) was added for incubation at room temperature for 30 minutes. The samples were then washed with PBS 3 times (2 min/time) again and observed under the microscope after developing by diaminobenzidine (DAB) (ZSGB-Bio Ltd., Beijing, China). With an appearance of tan precipitation, the reaction was withdrawn by running water. The cell nucleus was re-stained by Hematoxylin at the end of coloration. After washing and bluing by water, samples were dehydrated by conventional gradient alcohol, transparentized by xylene, and sealed in neutral gum. Staining was evaluated according to the proportion occupied by positive cell number in total cells. Three places were randomly selected within 10X vision in each sample. This was based on which ratio of positive cells was calculated and average values were regarded as the results of staining.

Follow up

Five-year follow ups were conducted for all patients that were discharged from hospital in the case group. 1, 3, and 5-year survival rates and prognosis recurrence including local neoplasm recurrence, distant metastasis, and new lesions were recorded. Follow ups were mainly performed by review and telephone, beginning from the day when the diagnosis was confirmed and ending up to 31st Dec. 2016, with an interval of 3 months. There were no cases lost to follow up.

Statistical analysis

Statistical analysis was conducted using the Statistical Package for Social Sciences (SPSS)
were analyzed by t-test. Categorical data were compared by χ² test. Receiver operating characteristic (ROC) curve was adopted to assess the diagnostic value of c-Jun, JNK, p-c-Jun, and p-JNK protein expressions for NPC. Survival analysis was evaluated by Kaplan-Meier and comparison of survival rates was performed by log-rank test. Level of significance was set at $P < 0.05$.

**Results**

*NPC patients and patients with chronic nasosinusitis are comparable with regards to age, gender, and smoking history*

Student’s t-test was employed to compare baseline information between case and benign control groups. The case group consisted of 122 patients, including 94 males and 28 females between 24-71 years old (with mean age of $48.43 ± 9.46$ years). TNM staging: 3 cases in stage I, 28 cases in stage II, 43 cases in stage III, and 48 cases in stage IV. Of the 122 patients, 10 patients had family history of NPC and 68 patients had a smoking history. The benign control group consisted of 136 patients, including 99 males and 37 females between 26-79 years old (with mean age of $49.12 ± 9.43$ years). Of these, 73 patients had a smoking history. In terms of age composition, gender ratio, and smoking history, no significant differences were found among these three groups (all $P > 0.05$). These results indicate that our study population was comparable.

**Activation of c-Jun/JNK signaling pathways contributes to occurrence of NPC**

c-Jun-, JNK-, p-c-Jun-, and p-JNK-positive cells were measured by immunohistochemistry staining to explore the role of c-Jun/JNK signal-
Expressions of c-Jun, JNK, p-c-Jun, and p-JNK achieve relatively good prediction for NPC.

ROC curve was adopted to assess the diagnostic values of c-Jun, JNK, p-c-Jun, and p-JNK protein expression for NPC. Results are shown in Figure 2. Areas under ROC curve of c-Jun, JNK, p-c-Jun, and p-JNK protein expression were 0.943, 0.968, 0.963, and 0.938, indicating that expression of c-Jun, JNK, p-c-Jun, and p-JNK proteins have medium value for diagnosing NPC. The maximum of Youden index was treated as our basis of selection. Cutoff value, sensitivity, and specificity of parameters are recorded in Table 2. These results demonstrate that expression of c-Jun, JNK, p-c-Jun, and p-JNK can be used as diagnostic factors for NPC.

Activation of c-Jun/JNK signaling pathways results in NPC progression

To explore correlation of c-Jun/JNK signaling pathways with clinical features of NPC, immunohistochemistry staining was used to measure c-Jun, JNK, p-c-Jun, and p-JNK protein expression. According to our results, c-Jun, JNK, p-c-Jun, and p-JNK protein expression was associated with TNM staging (all \( P < 0.05 \)) but failed to have obvious relationships with age, gender, smoking history, and family history of NPC (all \( P > 0.05 \)). Positive expression rates of these proteins were significant higher in patients with stage III-IV than those with I-II stage (all \( P < 0.05 \)). Additionally, c-Jun, JNK, p-c-Jun, and p-JNK protein expressions were positively correlated with TNM staging, indicating that positive expression rates were lower in patients with earlier stage, shown in Table 3. Thus, NPC progression is associated with activation of c-Jun/JNK signaling pathways.

Activation of c-Jun/JNK signaling pathways is unfavorable for NPC prognosis

To investigate correlation of c-Jun/JNK signaling pathways with NPC prognosis, immunohis-
Immunohistochemistry staining was adopted to detect c-Jun, JNK, p-c-Jun, and p-JNK protein expression. ROC curve, Kaplan-Meier, and log-rank test were employed to analyze survival rates of patients. Follow up was conducted on 122 patients and reoccurrence was found in 80 patients. Comparing c-Jun, JNK, p-c-Jun, and p-JNK protein expression between patients, with and without reoccurrence, we found that positive expression rates of these protein were higher in patients with reoccurrence than those without reoccurrence (Table 4).

The most favorable cutoff value of the ROC curve was adopted to divide patients into positive-expressed and negative-expressed groups, based on expression quantity (positive rate > cutoff value; negative rate < cutoff value). Kaplan-Meier method was applied to calculate the 1, 3, and 5-year survival rates (Figure 3). Mean survival time of dead patients is also presented in Table 5. According to these results, the 1, 3, and 5-year survival rates of different groups were as follows. c-Jun negative-expressed group: 100.0%, 88.2%, and 70.6%; c-Jun positive-expressed group: 73.3%, 57.1%, and 44.8%; JNK negative-expressed group: 100.0%, 90.0%, and 80.0%; JNK positive-expressed group: 75.0%, 58.9%, and 45.5%; p-c-Jun negative-expressed group: 100.0%, 92.9%, and 85.7%; p-c-Jun positive-expressed group: 74.1%, 57.4%, and 43.5%; p-JNK negative-expressed group: 100.0%, 97.8%, and 75.0%; p-JNK positive-expressed group: 73.6%, 56.6%, and 44.3%. Log-rank test indicated that 1, 3, and 5-year survival rates of patients in the c-Jun, JNK, p-c-Jun, and p-JNK positive-expressed groups were significantly lower than those in negative-expressed groups (all \( P < 0.05 \)). Comparing mean survival time of dead patients in the 5-year follow up, there were significant decreases of survival times.
c-Jun/JNK signaling pathways and NPC

in c-Jun, JNK, p-c-Jun, and p-JNK positive-expressed groups (all $P < 0.05$).

The above results indicate that activation of c-Jun/JNK signaling pathways plays a critical role in poor NPC prognosis.

Discussion

Currently, NPC is considered a disease with obvious geographic and ethnic discrepancies, commonly observed in Southern China, Northern Africa, and South Eastern Asia. Even with combined radiation and chemotherapy, the rate of recurrence is as high as 82% [15]. Unfortunately, treatment of most patients with loco-regionally advanced diseases will eventually fail with distant metastases [16]. Management of NPC is still difficult and challenging. There are arousing concerns about the requirement for improving early diagnosis and treatment of NPC based on failure cases of different stages [17]. This study was designed to elucidate the prognostic value of c-Jun/JNK signaling pathways in NPC and, hopefully, help to promote development of novel therapeutic treatments.

Initially, we found significant overexpression of c-Jun, JNK, p-c-Jun, and p-JNK proteins in NPC patients, in comparison to patients with benign NPC. In addition, ROC curve analysis was also conducted to testify the diagnostic significance of these proteins in NPC, indicating a medium diagnostic value of these proteins in NPC. As a

Table 5. Mean survival time of the dead patients negatively correlated with activation of c-Jun/JNK signaling pathways

<table>
<thead>
<tr>
<th>Items</th>
<th>Negative expression (M)</th>
<th>Positive expression (M)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-Jun</td>
<td>$54.76 \pm 12.42$</td>
<td>$38.45 \pm 22.66$</td>
<td>0.015</td>
</tr>
<tr>
<td>JNK</td>
<td>$57.27 \pm 7.54$</td>
<td>$39.08 \pm 22.54$</td>
<td>0.042</td>
</tr>
<tr>
<td>P-c-Jun</td>
<td>$57.67 \pm 6.51$</td>
<td>$38.35 \pm 22.62$</td>
<td>0.007</td>
</tr>
<tr>
<td>P-JNK</td>
<td>$57.38 \pm 6.4$</td>
<td>$38.21 \pm 22.69$</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Note: JNK, c-Jun N-terminal kinase; p-c-Jun, phospho-Ser63 c-Jun; p-JNK, phospho-Thr183/185 p-JNK; M, month.

Figure 3. The survival curve revealed that NPC patients with activated c-Jun/JNK signaling pathways have shorter survival. A. Survival curve of patients with c-Jun; B. Survival curve of patients with JNK; C. Survival curve of patients with p-c-Jun; D. Survival curve of patients with p-JNK; JNK, c-Jun NH 2-terminal kinases; NPC, nasopharyngeal carcinoma.
component of transcription factor nuclear-transcription-factor-activating protein-1 (AP-1), the proto-oncogene c-Jun plays a crucial role in various cellular processes such as growth, proliferation, and mediating tumor progression [18]. Julia et al. identified significant association between c-Jun-protein phosphorylation and activation of ERK 1/2 MAP kinase and Jun kinase in clinical breast cancers [19]. Chen et al. reported that serine/threonine phosphatase calcineurin (CaN) dephosphorylates C-terminus of c-Jun, promoting interaction between c-Jun and Sp1 and the subsequent increase of c-Jun-induced gene expression [20]. Evidence has been provided that increased expression of CaN could result in c-Jun-induced cell transformation and, thus, CaN increases the half-life of c-Jun proteins resulting in high expression of c-Jun, followed by enhancement of tumorigenesis [18]. Collectively, increases in cellular endpoints, including differentiation and cell apoptosis, may flow in any simple correlation within activation of c-Jun and proliferation. These diverse effects could also be a good explanation of the relationship observed between increase of activated c-Jun and poor prognosis [19].

Also, our results demonstrate that expression of c-Jun, JNK, p-c-Jun, and p-JNK proteins is positively correlated with TNM staging, indicating an earlier TNM stage correlates with lower positive expression rates of these proteins. As Chou et al. demonstrated, high survival rates of NPC patients have been reported for stage I and stage II, conforming with our results [15]. According to Edward et al., along with the development of androgen-insensitive prostate cancer (AIPC), patients with high levels of activated c-Jun survived shorter periods of time than patients in early stages of AIPC [21]. Interestingly, the relationship between increased c-Jun activation and prognosis of NPC is noted. We found that expression rates were higher in patients with recurrence. Marije et al. indicated a trend of worse survival due to an increase of c-Jun expression, signifying that c-Jun expression is connected to progression to a worse phenotype [22]. As the biological behavior of NPC depends on its nodal status, patients with advanced nodal disease are likely to have a poor outcome from chemotherapy and drug resistance may hamper the efficacy of anticancer drugs [23]. Furthermore, despite improving prognosis of NPC and a high cure rate for early stages through radiotherapy and chemotherapy, patients with locally advanced and metastatic NPC sometimes have unsuccessful outcomes and drug resistance may influence efficacy of anticancer drugs [24, 25]. However, the precise mechanism of c-Jun/JNK signaling pathways on clinical response to radiotherapy and chemotherapy for NPC patients remains unknown. Further exploration is required to pave the way for favorable prognosis for NPC patients.

In summary, overexpression of c-Jun, JNK, p-c-Jun, and p-JNK proteins has been discovered in patients with NPC, correlating with TNM staging and poor prognosis and suggesting that c-Jun/JNK signaling pathways can act as predictors for early diagnosis and prognosis. Therefore, our findings give rise to the intriguing possibility that therapies targeting JNK can contribute to prevention of relapse and/or metastasis of NPC.

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Disclosure of conflict of interest

None.

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c-Jun/JNK signaling pathways and NPC


