Original Article
Aberrant expression of Mas and iNOS in nasopharyngeal carcinoma and the clinical significance: an immunohistochemistry study

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Abstract: As an upstream activator for inducible nitric oxide synthase (iNOS), Mas has been reported to be up-regulated in several cancers. However, its role in human nasopharyngeal carcinoma (NPC) is still unclear. In this study, we performed the immunohistochemistry to detect the expression of Mas and iNOS in 115 cases of NPC and 30 cases of normal nasopharyngeal tissues, and investigated the relationship between these biomarkers and the relevant clinicopathological parameters. Mas expression in NPC tissues was significantly higher than that in normal nasopharyngeal tissues (P = 0.001). Overexpression of Mas was prominently related to lymphatic metastasis (P = 0.001) and advanced clinical stage (P = 0.039). Besides, the relative level of iNOS in NPC tissues was obviously higher than its expression in normal tissues (P < 0.001). High-level iNOS expression was also associated with older age (P = 0.005), lymphatic metastasis (P = 0.010) and advanced clinical stage (P = 0.003). Moreover, the result of spearman correlation test showed a significant positive relationship between Mas and iNOS (r = 0.300, P < 0.001). In ROC analysis, the confederative marker that combining Mas and iNOS had a more potent and efficient diagnostic value for NPC than either single makers (AUC = 0.782, 95% CI: 0.691-0.873, P < 0.001), which indicated the potential application of Mas and iNOS in clinical practice for screening and diagnosing metastatic nasopharyngeal carcinoma. In conclusion, Mas and iNOS are closely related to the carcinogenesis and development of NPC, indicating that these biomarkers could be served as potential prognostic indicators for NPC patients.

Keywords: Mas, iNOS, nasopharyngeal carcinoma, immunohistochemistry, ROC curve

Introduction
Nasopharyngeal carcinoma (NPC) is a specific cancer with ethnic and geographic distributions [1]. As reported, NPC is a leading form of cancer in North Africa and Southeast Asia, especially in Southern China [2-4]. An estimated 41,503 new cases and 20,058 deaths were attributed to NPC in China in 2010, accounting for 1.34% of all new cancer cases and 1.03% of all cancer-related deaths that year in China [5]. Due to the secretiveness of pathological changes, patients with NPC are often diagnosed at an advanced stage in primary hospitalization. Consequently, the survival rate for these patients has not been significantly improved yet even with the application of radiotherapy or chemotherapy. Thus, it is extremely necessary to elucidate the molecular mechanism underlying NPC pathogenesis and to explore effective biomarkers for early diagnosis and accurate prognosis, as well as novel therapeutic targets.

The G protein-coupled receptor Mas, which was identified as an Ang-(1-7) receptor [6], has been extensively studied in several types of cancer, such as colon adenocarcinoma [7], osteosarcoma [8], hepatocellular carcinoma [9], lung cancer [10, 11] and breast cancer [12, 13]. Recently, Pei et al reported that Mas was up-regulated in 26 cases of NPC specimens, and could mitigate proliferation and migration of NPC cells [14]. So far, however, there is no documentation of Mas expression with clinicopath-
ological parameters in NPC and, to the best of our knowledge, this is the first investigation using ROC curve analyses in large scale of cases of NPC tissues and the results indicated the potential value of Mas as a screening biomarker for NPC.

It has been widely studied that iNOS plays an important role in carcinogenesis [15], development [16], apoptosis [17], and cancer therapy [18]. Inducible NOS, or NOS2, is the key rate-limiting enzyme for generating nitric oxide in vivo [19]. NO has been found to be involved in tumorigenesis [20] and angiogenesis [21], which could promote the formation of invadopodia and increase the metastatic properties of NPC cells [16]. Previously, Li et al detected high expression levels of iNOS in NPC cells [22]. Dabbeche-Bouricha et al found higher expression of iNOS in nasopharyngeal tumors of Tunisian patients, compared with melanoma, and reported that expression of iNOS was correlated with age and clinical stage at diagnosis [23], but whether it can be used as a biomarker for predicting metastasis of nasopharyngeal carcinoma is still unknown, and the mechanism by which iNOS participates in cancer progression has not yet been fully established. This is the first study that reported the relationship of Mas and iNOS in NPC, and we also further expounded the clinical significance of the combined two markers in the current study.

Materials and methods

Patients population and histologic analysis

One hundred and fifteen cases of NPC tissues and thirty cases of normal nasopharyngeal tissues were collected in this retrospective study between March 2011 and December 2012. None of the patients recruited in this study had chemotherapy or radiotherapy before the biopsy. All samples obtained from biopsy were fresh-frozen in liquid nitrogen and stored at -80°C until further analysis. The pathological stage of each specimen was determined according to the guidelines of the International Union Against Cancer (UICC) staging system, and histopathology subtypes were determined based on the WHO classification [24]. All samples were independently reviewed and diagnosed by two pathologists (ZL and PL). The study protocol had been approved by the Ethical Committee of the First Affiliated Hospital of Guangxi Medical University. Written informed consent for using the samples for research was obtained from the patients and clinicians.

Clinicopathological information was obtained from medical records and summarized in Table 1. The male to female ratio was 72 to 43, and the median age was 52 years, ranging from 34 to 78 years. The number of NPC patients with stage I, II, III and IV was 3, 42, 40 and 30, respectively. With respect to the pathological subtypes, 52 cases were differentiated and 63 cases were undifferentiated. Until December 2014, 23 patients suffered recurrence or metastasis among a total of 115 candidates through 36 to 46 months’ follow-up, which, in detail, included 4 orthotopic recurrences, 6 distant metastases as well as 13 cases recurrent patients with metastases.

Immunohistochemistry

Immunohistochemistry was performed as previously reported [25, 26]. The routinely fixed paraffin-embedded blocks were sectioned at 4 μm thickness and then processed for immuno-histochemistry. Sections were deparaffinized, rehydrated and microwaved in 0.1 M citrate buffer for 10 min for antigen retrieval. After washing, slides were incubated in 3% H₂O₂ for 8 min to block the endogenous peroxidase activity, and next they were immunostained. Mas monoclonal antibody (present from Prof Wenzhen Lin, Department of Biochemistry, Guangxi Medical University) and iNOS monoclonal antibody (SC-651, Beijing Zhongshan Jinqiao Inc., Beijing, China) were incubated for 40 min at room temperature. PBS as a blank control replaced the initial antibody. Power Vision™ was performed in the immunostaining, according the guideline from manufacturer. High-temperature heat technique was used in antigen repair. Positive signaling was located in the membrane and cytoplasm of tumor cells, and the appearance of yellow or brown granule was defined as positive activation. The percentage of positive activation cell more than 10% was graded as positive expression.

Statistical analysis

SPSS 20.0 was used for data statistical analysis. Nonparametric Mann-Whitney U-test was prominently performed to analyze the significance of difference between two groups.
Mas and iNOS in NPC

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Total (n)</th>
<th>Expression of Mas n (%)</th>
<th>Z</th>
<th>P</th>
<th>Expression of iNOS n (%)</th>
<th>Z</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue</td>
<td></td>
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<tr>
<td>Normal tissue</td>
<td>30</td>
<td>26 (86.7%) 4 (13.3%)</td>
<td>-3.183</td>
<td>0.001</td>
<td>25 (83.3%) 5 (16.7%)</td>
<td>-3.960</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>NPC</td>
<td>115</td>
<td>63 (54.8%) 52 (45.2%)</td>
<td></td>
<td></td>
<td>49 (42.6%) 66 (57.4%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>43</td>
<td>24 (55.8%) 19 (44.2%)</td>
<td>-0.171</td>
<td>0.864</td>
<td>22 (51.2%) 21 (48.8%)</td>
<td>-1.427</td>
<td>0.153</td>
</tr>
<tr>
<td>Male</td>
<td>72</td>
<td>39 (54.2%) 33 (45.8%)</td>
<td></td>
<td></td>
<td>27 (37.5%) 45 (62.5%)</td>
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<td></td>
</tr>
<tr>
<td>Age (median)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>≤ 52</td>
<td>60</td>
<td>34 (56.7%) 26 (43.3%)</td>
<td>-0.422</td>
<td>0.673</td>
<td>33 (55.0%) 27 (45.0%)</td>
<td>-2.794</td>
<td>0.005</td>
</tr>
<tr>
<td>&gt; 52</td>
<td>55</td>
<td>29 (52.7%) 26 (47.3%)</td>
<td></td>
<td></td>
<td>16 (29.1%) 39 (70.9%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pathologic type</td>
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<td></td>
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<tr>
<td>Differentiated</td>
<td>52</td>
<td>29 (55.8%) 23 (44.2%)</td>
<td>-0.192</td>
<td>0.848</td>
<td>18 (34.6%) 34 (65.4%)</td>
<td>-1.568</td>
<td>0.117</td>
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<tr>
<td>Undifferentiated</td>
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<td>34 (54.0%) 29 (46.0%)</td>
<td></td>
<td></td>
<td>31 (49.2%) 32 (50.8%)</td>
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<td></td>
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<tr>
<td>Lymphatic metastasis</td>
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<tr>
<td>No</td>
<td>52</td>
<td>34 (65.4%) 18 (34.6%)</td>
<td>-2.066</td>
<td>0.039</td>
<td>29 (55.8%) 23 (44.2%)</td>
<td>-2.582</td>
<td>0.010</td>
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<tr>
<td>Yes</td>
<td>63</td>
<td>29 (46.0%) 34 (54.0%)</td>
<td></td>
<td></td>
<td>20 (31.7%) 43 (68.3%)</td>
<td></td>
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<td>Clinical stage</td>
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<tr>
<td>I-II</td>
<td>45</td>
<td>33 (73.3%) 12 (26.7%)</td>
<td>-3.191</td>
<td>0.001</td>
<td>27 (60.0%) 18 (40.0%)</td>
<td>-3.011</td>
<td>0.003</td>
</tr>
<tr>
<td>III-IV</td>
<td>70</td>
<td>30 (42.9%) 40 (57.1%)</td>
<td></td>
<td></td>
<td>22 (31.4%) 48 (68.6%)</td>
<td></td>
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<td>Recurrence</td>
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<tr>
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<td>51 (55.4%) 41 (44.6%)</td>
<td>-0.280</td>
<td>0.780</td>
<td>40 (43.5%) 52 (56.5%)</td>
<td>-0.376</td>
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<tr>
<td>Yes</td>
<td>23</td>
<td>12 (52.2%) 11 (47.8%)</td>
<td></td>
<td></td>
<td>9 (39.1%) 14 (60.9%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Relationship between Mas and iNOS expression and clinicopathological features

Spearman rank correlation test was used to analyze the correlation between Mas and iNOS expression and different parameters. Receiver operating characteristic (ROC) curves were plotted to evaluate the power of biomarkers to distinguish the NPC patients from non-cancerous nasopharyngeal tissue, as well as to predict some clinical features, including the status of lymph node metastasis, pathologic type and clinical stages. All statistical significance was determined at a level of P < 0.05.

Results

Mas expression in nasopharynx normal and cancer tissues

Mas was found to be highly expressed in the NPC tissues (45.2%, 52/115), whereas it was lowly expressed in the normal nasopharynx tissues (13.3%, 4/30, P = 0.001) (Table 1; Figures 1, 3A-F). In addition, ROC curve was performed to prove the diagnostic value of Mas in NPC patients. The area under curve (AUC) of Mas was 0.659 (95% CI: 0.559-0.760, P = 0.007). With respect to the association between Mas expression and clinicopathological features, the expression of Mas in the patients with lymphatic metastasis (54.0%, 34/63) was significantly increased compared to those without lymphatic metastasis (34.6%, 18/52, P = 0.039) (Figure 1). But the AUC of Mas for predicting lymphatic metastasis was 0.579 (95% CI: 0.493-0.701, P = 0.075), which was short of statistical significance. Moreover, the expression of Mas in advance stage (III-IV, 57.1%, 40/70) was significantly higher than that in early stage (I-II, 26.7%, 12/45, P = 0.001) (Figure 1), and the AUC to predict clinical stage was 0.652 (95% CI: 0.550-0.755, P = 0.006). According to Spearman correlation test, Mas aberrant expression was associated with lymphatic metastasis (r = 0.194, P = 0.038) and clinical stage (r = 0.299, P = 0.001). Nevertheless, no significant correlations were found between Mas expression and patient gender, age, pathologic type and tumor recurrence.

iNOS expression in nasopharyngeal normal and cancer tissues

The expression of iNOS significantly increased in NPC tissues (57.4%, 66/115) as compared to
Mas and iNOS in NPC

![Graph](image1.png)

**Figure 1.** Relationship between Mas expression and clinicopathological features. *P < 0.05; **P < 0.01; ***P < 0.001.

![Graph](image2.png)

**Figure 2.** Relationship between iNOS expression and clinicopathological features. *P < 0.05; **P < 0.01; ***P < 0.001.

the normal nasopharynx tissues (16.7%, 5/30, P < 0.001) (Table 1; Figure 2). Furthermore, the AUC of iNOS for diagnosing NPC was 0.704 (95% CI: 0.605-0.802, P = 0.001). In terms of the clinicopathological features of NPC, Mas was highly expressed in the patient older than median age (70.9%, 39/55), but less expressed in the patient younger than median age (45.0%, 27/60, P = 0.005), and received an AUC value of 0.630 (95% CI: 0.527-0.732, P = 0.017). The relative level of iNOS in NPC patients with lymphatic metastasis (68.3%, 43/63) was prominently higher than those without lymphatic metastasis (44.2%, 23/52, P = 0.010) (Figure 2), and the AUC was 0.602 (95% CI: 0.516-0.724, P = 0.027). Compared to patients with early stages (I-II, 40.0%, 18/45), the relative level of iNOS in those of advanced stages (III-IV, 68.6%, 48/70, P = 0.003) (Figure 2) was markedly increased. Simultaneously, the ROC curve in predicting clinical stage showed an AUC 0.643 (95% CI: 0.538-0.748, P = 0.010). Spearman analyze suggested a desirable correlation coefficient between iNOS expression and age (r = 0.262, P = 0.005), lymphatic metastasis (r = 0.242, P = 0.009) and clinical stage (r = 0.282, P = 0.002). However, no significant correlation was found between iNOS expression and the rest clinicopathological parameters.

**Combined application of Mas and iNOS in nasopharynx normal and cancer tissues**

Remarkable statistical meanings of Mas and iNOS were found in nasopharynx normal and cancer tissues. Either Mas or iNOS was positively expressed in the NPC tissues (73.0%, 84/115, P < 0.001) (Table 2), while negatively expressed in the normal nasopharynx tissues (83.8%, 25/30, P < 0.001) (Table 2). Additionally, proved by ROC method, the confederative marker that combining Mas and iNOS had a more potent and efficient diagnostic value for NPC than either single marker (AUC = 0.782, 95% CI: 0.691-0.873, P < 0.001). For clinicopathological parameters mentioned above, the combining markers showed a more significant value of differential expression in inflammatory or cancerous nasopharyngeal tissues. The expression of the combined markers was positive in patients with advanced age (83.6%, 46/55), lymphatic metastasis (88.9%, 56/63), advanced clinical stage (III-IV, 88.6%, 62/70), while negatively expressed in patients with low age (36.7%, 22/60, P = 0.015), non-metastasis of lymph node (46.2%, 24/52, P < 0.001), and early stage (I-II, 51.1%, 23/45, P < 0.001). Besides, the integrated marker also showed a higher AUC value to predict lymphatic metastasis condition (AUC = 0.675, 95% CI: 0.574-0.777, P = 0.001), and clinical stage (AUC = 0.698, 95% CI: 0.595-0.802, P = 0.010).
Mas and iNOS in NPC

Moreover, the results of Spearman correlation test showed a significant positive relationship between Mas and iNOS \( (r = 0.300, P < 0.001) \) in current study cohort. Further, the aberrant expression of combined use of Mas and iNOS was associated with age \( (r = 0.229, P = 0.014) \), lymphatic metastasis \( (r = 0.393, P < 0.001) \) and clinical stage \( (r = 0.436, P < 0.001) \). No significant correlation was found between Mas or iNOS expression and the rest clinicopathological parameters.

**Discussion**

Previous studies have demonstrated that the G protein-coupled receptor Mas can bind to and activate angiotensin (Ang) 1-7 [27], conferring anti-proliferative effects [8] and inducing tumorigenic transformation [28]. However, no clinical studies on clinicopathological features of Mas in NPC have been reported yet. In this study, we demonstrated that Mas was significantly highly expressed in the NPC tissues, and lowly expressed in corresponding normal tissues, which was consistent with the study of Pei et al [14]. Moreover, to further clarify the role of Mas in NPC, we performed immunohistochemical method to confirm a significant increase of Mas expression in patients with lymphatic metastasis and patients in advanced stage. From the perspective of statistics, the results of correlation analysis also indicated an intensely positive correlation between elevated Mas expression and lymphatic metastasis as well as clinical stage.

Overexpression of iNOS in nasopharyngeal carcinoma has also been reported in several studies [17, 20, 29, 30]. It was reported that expression of iNOS induced nitrotive and oxidative stress in NPC, which may promote the formation of 8-OHdG and 8-NitroG, leading to the origination and progression of NPC [29]. Ridnour et al has reported that tumors with highly expressed iNOS behave more aggressively via mechanisms that favor Akt pathway activation [31]. NOS activity, measured in specimens from the tumor periphery, has also been found to correlate strongly with lymphatic vessel density, lymphatic vessel area and expression of vascular endothelial growth factor-C in head and neck squamous cell carcinomas, indicating that iNOS activity may promote lymphangiogenesis and metastasis to lymph nodes in HNSCC [32]. Besides, it has been shown that iNOS expres-
sion may be a potential hallmark for predicting the outcome of radiotherapy in NPC patients [17], but whether it can be used as a biomarker for predicting metastasis of nasopharyngeal carcinoma is still unknown, and the mechanism by which iNOS participates in cancer progression has not yet been fully established. In line with pre-existing studies, we observed a significant association between elevated iNOS expression and lymphatic metastasis as well as advanced clinical stage in patients with NPC. Moreover, ROC curve analysis showed that expression of iNOS could contribute to early detection of NPC, discriminate lymphatic metastasis and advanced stage from the controls, giving an AUC of 0.704, 0.602 and 0.643, respectively.

Growing evidence indicates that activation of Mas is associated with the release of nitric oxide [33]. Yang et al also reported that Mas is highly expressed in relation to iNOS in differentiated catecholaminergic neurons [34]. In this study, Mas and iNOS were co-overexpressed in patients with NPC. Both Mas and iNOS had significant diagnostic value for NPC and yielded AUC of 0.659 and 0.602, respectively. Combined ROC analyses with these two targets could yield an increased AUC of 0.782 in discriminating NPC from normal controls. More importantly, for clinicopathological parameters mentioned above, the combining markers showed a more significant value of differential expression between inflammatory and cancerous nasopharyngeal tissues. The aberrant expression of combined use of Mas and iNOS was significantly associated with age (r = 0.229, P = 0.014), lymphatic metastasis (r = 0.393, P < 0.001) and clinical stage (r = 0.436, P < 0.001), suggesting their potential value for prognostic detection of NPC. But the mechanisms of this enhancement require further investigation.

In summary, our findings demonstrate that Mas and iNOS are positively expressed in patients with NPC. We notice a tender for Mas and iNOS to express at higher levels after lymphatic metastasis occurs in patients with NPC. Mas and iNOS may serve as useful biomarkers for the diagnosis and prognosis of NPC. The combined use of Mas and iNOS could improve their utility in screening and diagnosing metastatic nasopharyngeal carcinoma. Further investigations are needed to confirm a link between Mas and iNOS in NPC.

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Disclosure of conflict of interest

None.

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