Increased expression of monoamine oxidase A is associated with epithelial to mesenchymal transition and clinicopathological features in non-small cell lung cancer

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Abstract. Monoamine oxidase A (MAOA), a mitochondrial enzyme, is closely associated with neurological disorders. Recently, MAOA has been linked to the progression of prostate cancer, hepatocellular carcinoma, and cholangiocarcinoma. However, MAOA was reported to have different effects on the progression of these types of cancer, and the role of MAOA in non-small cell lung cancer (NSCLC) progression remains unclear. The present study determined the expression of MAOA and epithelial to mesenchymal transition (EMT) markers in 45 pairs of NSCLC and matched non-tumor adjacent lung tissues, and further analyzed the correlation between MAOA expression and the EMT or the development of clinicopathological features. The results demonstrated that protein and mRNA expression levels of MAOA in NSCLC tissues were higher than those observed in the matched non-tumor adjacent lung tissues. Furthermore, the increased MAOA expression in NSCLC tissues was positively correlated with N-cadherin (r=0.525, P=0.002), Slug (r=-0.515, P=0.001), and Twist (r=0.448, P=0.008) expressions, but negatively correlated with E-cadherin expression (r=-0.387, P=0.01). Additionally, the elevated MAOA expression in NSCLC tissues was associated with late stage NSCLC (Z=-2.596, P=0.029) and lymph node metastases (Z=-2.378, P=0.020). These findings suggest that MAOA may have a role in promoting NSCLC progression by mediating EMT.

Introduction

Lung cancer, the leading cause of cancer-related deaths worldwide, is commonly divided into two categories, small cell lung cancer (SCLC) and non-SCLC (NSCLC), depending on its degree of differentiation and morphological characteristics (1,2). NSCLC accounts for ~80% of primary lung cancers, including squamous cell carcinoma, adenocarcinoma, and large cell carcinoma (3). The 5-year survival rate of NSCLC is only 7% (4). Moreover, lymph nodes and distant organ metastasis are the main reasons leading to treatment failure in NSCLC patients with radical resection (5,6).

Epithelial to mesenchymal transition (EMT), a reversible biological process, is characterized by the loss of epithelial cell junction proteins (E-cadherin, ZO-1) (7,8), the gain of mesenchymal markers (vimentin, N-cadherin) (9,10), and the activation of transcription factors (Snail1, Slug, ZEB1, Twist) (11-15). Accumulating evidence indicates that EMT enhances tumor invasion, distant metastasis, and chemoresistance in NSCLC, underscoring the need for a comprehensive understanding of the EMT function in NSCLC progression (16-19).

Monoamine oxidase A (MAOA), a mitochondria-bound enzyme, catalyzes the oxidative deamination of dietary amines and monoamine neurotransmitters, such as serotonin, norepinephrine, and dopamine (20,21). The functions of MAOA have been extensively studied in the context of neurological disorders, including mental depression, aggressive behaviors, and Parkinson's disease (22,23). Recent studies have indicated the role of MAOA in the progression of prostate cancer (24-30), hepatocellular carcinoma (HCC)(31), and cholangiocarcinoma (32). High Gleason grade or poorly differentiated prostate cancer exhibited increased MAOA expression (24), and the increased MAOA promoted prostate cancer metastasis (25,26). Furthermore,

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overexpression of MAOA was found to dramatically down-regulate the expression of E-cadherin and upregulate the expression of vimentin and Twist at both mRNA and protein levels in prostate cancer (25). These studies suggested that MAOA might promote the progression of prostate cancer by mediating EMT. However, conflicting results were reported for HCC (31) and cholangiocarcinoma (32). Therefore, the role of MAOA may vary across cancer types, and therefore, it is essential to further understand the function of MAOA in other cancers.

Little is known about the function of MAOA in NSCLC. Accordingly, in this study, we investigated the expression of MAOA in NSCLC tissues and analyzed the association between the expression of MAOA and EMT or the development of clinicopathological features. We found for the first time, to the best of our knowledge, that MAOA protein and mRNA expressions in NSCLC tissues were significantly higher than those observed in the matched non-tumor adjacent lung tissues, and the increased MAOA expression was related to EMT, clinical stages, and lymph node metastases in NSCLC, suggesting that MAOA may be involved in mediating the progression of NSCLC.

Materials and methods

Reagents. Rabbit anti-human MAOA monoclonal antibody was obtained from Abcam (ab126751; Cambridge, UK). Mouse anti-human E-cadherin monoclonal antibody, rabbit anti-human vimentin, N-cadherin, Snail1, Slug, Zo-1, ZEB1 and Twist monoclonal antibodies, and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). The RNA extraction kit (RNAsplit Pure FFPE kit) was purchased from Tiangen Biotech Co., Ltd. (Beijing, China). The reverse transcription (RT) kit (PrimeScript™ RT reagent kit) and qPCR analysis kit (SYBR Premix Ex Taq™ II) were obtained from Takara Biotechnology Co., Ltd. (Dalian, China).

NSCLC patients and control cases. NSCLC tissue specimens were obtained from 45 patients who were definitively diagnosed with NSCLC and had undergone curative surgery between 2007 and 2010 at the Affiliated Hospital of Guangdong Medical University (Guangdong, China). The matched non-tumor adjacent lung tissues (1 cm from the tumor) were also collected from the same patients, as the controls. Among these patients, the complete clinicopathological and histopathological data were collected from 30 cases. The patients that met the following criteria were enrolled. First, the patients were definitively diagnosed with NSCLC based on histological examinations. Second, the patients had not received chemotherapy, immunotherapy, or radiotherapy before pulmonary lobectomy. Third, the patients showed normal hepatic and renal functions and no abnormality of the endocrine system.

Ethics approval. Either the patients or their close relatives provided informed written consents. Our investigation received the ethic approval from the local Committee of the Affiliated Hospital of Guangdong Medical University. All clinical investigations were performed according to the principles defined by the Declaration of Helsinki.

Immunohistochemistry. Immunohistochemical staining was performed on paraffin-embedded tissue specimens, including NSCLC and matched non-tumor adjacent lung tissues, from 45 cases. Briefly, paraffin-embedded tissue specimens were cut into 4-µm sections, transferred onto Superfrost Ultra Plus slides, and placed in a 60°C oven overnight. The slides were deparaffinized with xylene, rehydrated in a descending alcohol series (100, 95, 90, 80 and 70%), and then rinsed with sterile distilled water for 5 min. Antigen retrieval was performed by boiling the tissue sections in citrate buffer (10 mM trisodium citrate, 0.05% Tween-20, pH=6) for 10 min. The slides were further treated with 3% hydrogen peroxide for 15 min to inactivate any endogenous peroxidase activity. After rinsing in phosphate-buffered saline (PBS), non-specific sites were blocked with normal goat serum for 15 min. The slides were subsequently incubated with primary antibodies (1:100) overnight at 4°C. One slide was incubated with PBS, as the negative control. After washing with precooled PBS, the slides were incubated with a secondary biotinylated antibody for 15 min at room temperature. The slides were then stained with diaminobenzidine (DAB), and counterstained with hematoxylin, before being observed and analyzed in a double-blind manner, under light microscopy, by two senior pathologists. Ten randomly selected fields were examined at x400 magnification and 100 cancer cells were counted in each field (total 10,000 cells) to determine the proportion of positive cells. A semi-quantitative analysis was performed to evaluate the protein expression levels as described previously (33,34). In brief, staining intensity was scored on a scale of 0 to 3, 0 for no intensity, 1 for low intensity (light yellow), 2 for moderate intensity (claybank), and 3 for high intensity (sepia). The cell positivity was scored on a scale of 0 to 4: 0, <5% cells stain-positive; 1, 5 to 25% cells stain-positive; 2, 26 to 50% cells stain-positive; 3, 51 to 75% cells stain-positive; and 4, >75% cells stain-positive. The scores obtained relative staining intensity and proportion of positive cells were multiplied together to generate a final score ranging from 0 to 12, interpreted as follows: 0, negative (-); 1 to 4, weakly positive (+); 5 to 8, moderately positive (++); 9 to 12, strongly positive (++++). The scores were evaluated by two pathologists.

RT-qPCR. Total RNA was extracted from paraffin-embedded tissue specimens using the TIANGEN RNAsplit Pure FFPE kit and then converted to cDNA using the PrimeScript™ RT reagent kit (both from Tiangen Biotech Co., Ltd.). qPCR analysis was performed using SYBR Premix Ex Taq™ II (Takara Biotechnology Co., Ltd., according to the manufacturer’s instructions. All the primers were synthesized by Takara Biotechnology Co., Ltd., and are listed in Table I. The housekeeping gene β-actin was used as an internal control to normalize mRNA levels. The optimum reaction conditions for qPCR were as follows: Pre-treatment at 42°C for 5 min, initial denaturation at 95°C for 10 sec, followed by 40 cycles at 95°C.
for 5 sec, and 60°C for 31 sec. The experiment was carried out in triplicate.

Statistical analysis. SPSS 19.0 Windows software was used for statistical analysis. Quantitative data were presented as the mean ± SD. The categorical variables were presented as frequency and percent rates, and the positive rates from two groups were compared using Chi-square (χ²) test or Fisher exact probabilities (n<40 or T<1). Wilcoxon rank sum test was used to perform the statistical analysis on ordinal data. Spearman rank correlation coefficient was employed for the correlation analysis. P-value <0.05 was considered to indicate a statistically significant difference.

Results

Expressions of MAOA and EMT markers in NSCLC and matched non-tumor adjacent lung tissues. Previous studies have demonstrated that prostate cancer and HCC exhibit completely different MAOA expression levels (24,31), and to date, MAOA expression has not been reported in NSCLC. To investigate the expressions of MAOA and EMT markers in NSCLC tissues, immunohistochemical staining was performed in 45 pairs of NSCLC and patient-matched non-tumor adjacent lung tissues. The protein expressions of MAOA, N-cadherin, vimentin, Snail1, Slug, ZEB1, and Twist were obviously enhanced in NSCLC tissues (Fig. 1A). The positive expression rates of MAOA, N-cadherin, vimentin, Snail1, Slug, ZEB1, and Twist in NSCLC tissues were higher than those observed in the matched non-tumor adjacent lung tissues (P<0.01, Fig. 1B; Table II), while the positive expression rates of E-cadherin, Zo-1, and EMT epithelial makers determined in NSCLC tissues were lower than those observed in adjacent normal lung tissues (Fig. 1A and B; Table II).

To further investigate the mRNA expressions of MAOA and EMT markers, NSCLC and matched adjacent normal lung tissues from 30 cases were selected for RT-qPCR. Our results showed that the mRNA levels of MAOA, N-cadherin, vimentin, Snail1, Slug, ZEB1, and Twist in NSCLC tissues were significantly higher than those detected in non-tumor adjacent tissues. As expected, a significant decrease in E-cadherin and Zo-1 mRNA expressions was observed in NSCLC tissues (P<0.01; Fig. 1C).

Moreover, the statistical distribution results further demonstrated that MAOA, vimentin, and Snail1 protein expressions were significantly stronger in NSCLC tissues than in adjacent normal lung tissues, whereas E-cadherin protein expression displayed the opposite trend (P<0.05; Fig. 2).

Correlation between the expressions of MAOA and EMT markers in NSCLC. To study the role of MAOA expression in the EMT of NSCLC, a Spearman rank correlation coefficient test was performed to analyze the correlation between the expressions of MAOA and EMT markers in NSCLC. As described in Table III, although there was no relationship between the expression of MAOA and the expressions

<table>
<thead>
<tr>
<th>GenBank no.</th>
<th>Genes</th>
<th>Primer sequence (5'-3')</th>
<th>Length (bp)</th>
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</table>
| NM_000240.3 | MAOA  | Forward primer AGTGAGCGAAGGATAATGG  
Reverse primer TGGTCATGGTTCACGCTTC  | 114 |
| NM_004360.4 | E-cadherin | Forward primer TTGCTACTGGAACAGGAGCAG  
Reverse primer CCCGTGTATGTCAGTGTGT  | 179 |
| NM_003257.4 | Zo-1  | Forward primer GGATGTTTATCGTCGCAATTGTA  
Reverse primer AAGAGCCCAGTTTTCCATTTGTA  | 158 |
| NM_001792.4 | N-cadherin | Forward primer TATATCTTTGCTGTAGTGTGT  
Reverse primer TCTTCCTTCTCCTCCACCTTCCTTC  | 139 |
| NM_003380.3 | vimentin | Forward primer TGGGCACGTCTTACCTTGAA  
Reverse primer GGCATCGGTGACCTGAGAA  | 176 |
| NM_005985.3 | Snail1 | Forward primer TCCCTTCGTCTCCCTCACTT  
Reverse primer TGGTCAGATTTCGGCAGTGAAG  | 155 |
| NM_003068.4 | Slug | Forward primer GCCCTTTTTTTCGCTCAC  
Reverse primer GGTATGAGGTGAGTTCACGCTTCAC  | 115 |
| NM_030751.5 | ZEB1 | Forward primer TCCCCATACCTCTAACCCTT  
Reverse primer CCCGTGTATGTCAGTGTGT  | 122 |
| NM_005474.3 | Twist | Forward primer AGTCCGACATCGACCTGAGGAG  
Reverse primer GACCTAGTTAGGAAAGTCTGAGT  | 146 |
| NM_001101.3 | β-actin | Forward primer TGACGTGGACATCCGGCAAG  
Reverse primer CTGGAAGGTGACAGCAGG  | 186 |

MAOA, monoamine oxidase A.
of vimentin, Snail1, Zo-1, or ZEB1 (P>0.05; Table III), the expression of MAOA was positively correlated with the expressions of the EMT mesenchymal marker N-cadherin (r=0.525, P=0.002) and the EMT transcription factors Slug (r=0.515, P=0.001) and Twist (r=0.448, P=0.008). Accordingly, MAOA expression was negatively correlated with the expression of the epithelial maker E-cadherin (r=-0.387, P=0.01; Table III).

**Correlation between MAOA expression and the development of clinicopathological features in NSCLC.** Thirty of the patients who were definitively diagnosed with NSCLC, and
offered complete clinicopathological and histopathological data, were enrolled to analyze the correlation between MAOA expression and the clinicopathological features observed in NSCLC. A Wilcoxon rank sum test was employed to carry out the analysis of statistical distribution on the data obtained regarding the protein expression levels. Our results showed that the positive rate of MAOA expression in stage III was higher than that measured in stages I and II (Z= -2.596, P=0.029; Table IV). Additionally, the lymph node metastasis group exhibited a stronger MAOA expression level than the controls with no metastasis (Z= -2.378, P=0.020; Table IV). These results indicated that MAOA expression was significantly correlated with clinical stages and lymph node metastases. However, MAOA expression was not influenced by sex, age, degree of differentiation, or histological types (P>0.05; Table IV).

Discussion

MAOA, a monoamine neurotransmitter degrading enzyme, is well-known to be closely associated with impulsive aggressively, anxiety, depression, among other emotions, and is considered as an indicator of psychological status (22,23,35). Recently, several studies have been focusing on the relationship between MAOA expression and cancers (24-32). However, conflicting results were reported across different types of cancer, including prostate cancer (24-30), HCC (31), and cholangiocarcinoma (32). MAOA was demonstrated as being highly expressed in high-grade aggressive prostate cancer, and capable of mediating prostate tumorigenesis and metastasis (24-27). Recently, MAOA was reported as a novel decision maker in apoptosis and autophagy processes occurring within hormone refractory neuroendocrine prostate cancer cells (28). Moreover, clorgyline, a MAOA inhibitor, was found to exhibit anti-oncogenic and pro-differentiation effects on high-grade prostate cancer cells (29), and the MAOA inhibitor-near-infrared dye conjugate was reported to reduce prostate tumor growth (30). These findings suggest that MAOA might play a key role in mediating prostate cancer progression. However, Li et al demonstrated that MAOA expression was remarkably downregulated in clinical HCC tissue samples (31), and that MAOA suppressed HCC metastasis by inhibiting the adrenergic system and its transactivation of EGFR signaling (31). Huang et al also found that MAOA expression was inhibited by coordinated epigenetic and IL-6-driven events in human cholangiocarcinoma (32), and that overexpression of MAOA suppressed cholangiocarcinoma growth and invasion (32). In the present study, we demonstrated for the first time to our knowledge that MAOA protein and mRNA expression levels, positive rates, and statistical distribution in NSCLC tissues were dramatically higher than those recorded in the matched non-tumor tissues.
adjacent lung tissues (Figs. 1 and 2; Table II). Moreover, we further found that MAOA expression was correlated with clinical stages and lymph node metastases, while no relation could be established with sex, age, degree of differentiation, and histological types (Table IV). Taken together, our results suggest that MAOA may play a role in promoting the progression of NSCLC.

EMT, a key step in invasion and metastasis, plays a crucial role in the progression of cancers, including NSCLC (16-19). Wu et al (25) demonstrated that MAOA expression in prostate cancer suppressed epithelial phenotype and promoted mesenchymal transition by decreasing the expression of epithelial marker E-cadherin, while increasing the expressions of mesenchymal marker vimentin and transcription factor Twist, indicating its association with EMT in prostate cancer. In the present study, we showed that the increased MAOA expression in NSCLC tissues was negatively correlated with E-cadherin expression, but positively correlated with the expressions of N-cadherin, Slug, and Twist (Table III), suggesting that MAOA may mediate EMT, leading to the progression of NSCLC.

In conclusion, we demonstrated for the first time, to the best of our knowledge, that MAOA expression was significantly increased in NSCLC tissues, which was positively associated with EMT, late stages and lymph node metastases of the cancer, thus supporting the notion that MAOA may play a role in NSCLC progression by regulating the EMT process.

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References