RESEARCH ARTICLE

Expression of High Mobility Group Box - B1 (HMGB-1) and Matrix Metalloproteinase-9 (MMP-9) in Non-small Cell Lung Cancer (NSCLC)

Jing-Luan Wang[1,2], Da-Wei Wu[1]*, Zhao-Zhong Cheng[2], Wei-Zhong Han[2], Sheng-Wei Xu[3], Ni-Na Sun[2]

Abstract

Objective: This study evaluated the expression level of high mobility group box-B1 (HMGB-1) and matrix metalloproteinase-9 (MMP-9) in non-small cell lung cancer (NSCLC) inmorder to reveal any relation with development and prognosis. Methods: NSCLC and normal tissues were selected from 30 patients at age of 30-73, and used for RT-PCR and Western blot analyses of HMGB-1. A total of 100 paraffin embedded NSCLC tissues were also isolated from patients through surgical resection, and used for detection of HMGB-1 by immunohistochemistry. In addition, 50 samples were also applied for MMP-9 detection, and 30 normal tissues were considered as controls. Correlation analysis of HMGB-1 and MMP-9 was carried out by Pearsons correlation coefficient. Results: The average expression level of HMGB-1 in NSCLC patients was significantly higher than in normal lung tissues. In addition, patients in III-IV period exhibit significantly higher positive rate of HMGB-1 when compared with I-II period cases. Furthermore, a positive correlation with HMGB-1 was found in the expression of MPP-9. Conclusion: HMGB-1 was highly expressed in NSCLC, which may become a prognostic and predictive marker for NSCLC. Besides, MPP-9 was positively correlated with HMGB-1.

Keywords: Non-small cell lung cancer - HMGB-1 - MMP-9

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Introduction

Lung cancer has currently become one of the most serious malignant tumor which will lead to cancer death (Salgia, 2011). Non-small cell lung cancer (NSCLC) comprises about 80-85% of all lung cancers, which include three major types: adenocarcinoma, epidermoid carcinoma, and large-cell carcinoma (Ettinger et al., 2008). In decades since the last Lancet Seminar on lung cancer, there have been advances in many aspects of the classification, diagnosis, and treatment in NSCLC (Goldstraw et al., 2011). However, the knowledge of pathogenesis in NSCLC is still limited, and the effective indicators for early diagnosis and prognosis of NSCLC were lacked. As known, early diagnosis is thought to be an important means to reduce the mortality of NSCLC. Therefore, looking for effective indicators for early diagnosis and prevention of lung cancer become a focus on recent research.

Several biomarkers have emerged as prognostic and predictive markers for NSCLC, such as epidermal growth factor receptor (EGFR), the 5' endonuclease of the nucleotide excision repair complex (ERCC1), K-ras oncogene, and the regulatory subunit of ribonucleotide reductase (RRM1) (Ettinger et al., 2010). As reported, EGFR is detecta¬ble in approximately 80% to 85% of patients with NSCLC, and the expression level was varied widely on a continual scale (Nomoto et al., 2006). ERCC1 can be found in all tumor cells, and its expression level was also varied widely. In completely resected NSCLC of patients who did not un¬dergo preoperative chemotherapy or radiation, the ERCC1 mRNA levels were prognostic of survival. Patients who had high expression level of ERCC1 would lived significantly longer than patients whose tumors had low levels (Simon et al., 2005; Olaussen et al., 2006). K-ras is a GTP-binding protein and involved in G-protein–coupled receptor signaling. In its mutated form, it is constitutively active, able to transform immortalized cells, and promotes cell proliferation and survival (Rodenhuis et al., 1987). Current data suggest that approximately 25% of adenocarcinomas in north American population have K-ras mutations (Eberhard et al., 2005; Tsao et al., 2007; Miller et al., 2008). RRM1 can also be found in all tumor cells, and its ex¬pression level was similar with ERCC1. Patients with high expression of RRM1 had a median overall survival of greater than

*[1]Department of Critical Care Medicine, Qilu Hospital, Shandong University, Jinan, [2]Department of Respiratory Medicine, The Affiliated Hospital of Qingdao University, [3]Department of Respiratory Medicine, The Third People's Hospital of Qingdao, Qingdao, China *For correspondence: wdw.55@163.com*

120 months compared with 60 months for pa¬tients with low RRM1 expression (Bepler et al., 2004; Zheng et al., 2007).

High mobility group box-B1 (HMGB-1) is a highly conserved non-histone protein. It was involved in many important biological processes, such as transcription, DNA preparation, cell growth and differentiation, and extracellular signal transduction (Ellerman et al., 2007; Liu et al., 2010). HMGB-1 generally locates in the cell nucleus but is transported to cytoplasm in clear cell renal cell carcinoma (Wu et al., 2013). Overexpression of HMGB-1 may be an important biomarker for T-cell lymphoma (Mao et al., 2012). HMGB-1 was also reported to associate with reproductive differentiation and migration of tumor cells (Brezniceanu et al., 2003; Sasahira et al., 2008; Sims et al., 2009). Matrix metalloproteinase-9 (MMP-9) was mainly involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling. It was also reported to play a role in tumor-associated tissue remodeling. MMP-9 has important prognostic value for a variety of cancers, such as gastric cancer (Zhang et al., 2012; Gao et al., 2013), breast cancer (Song et al., 2013; Lin et al., 2014), ovarian cancer (Li et al., 2013) and cervical carcinoma (Wang et al., 2013). In this study, the expression of HMGB-1 in NSCLC and normal tissues was detected by RT-PCR, Western blot and immunohistochemistry. Besides, MMP-9 was also detected by immunohistochemistry. The correlation between HMGB-1 and MMP-9 was revealed.

Materials and Methods

Patients and Samples

In total 30 patients at age of 30-73 with NSCLC (19male, 11female) were selected from the affiliated hospital of Qingdao University between January, 2013 and March, 2013. Chronic obstructive pulmonary disease was excluded in this study. NSCLC tissues and normal tissues (more than 5cm from the edge of tumor) were isolated and stored at -70°C, which were used for RT-PCR and Western blot analyses of HMGB-1. Besides, 100 paraffin embedding NSCLC tissues used for detection of HMGB-1 by immunohistochemistry were isolated form patients through surgical resection between 2010 and 2012. In which 50 samples were also used for MMP-9 detection. 30 normal tissues were considered to be control (Table 1). This study was approved by the local ethics committee and conducted with written informed consent from the patients.

Table 1. The Number of Various Patients Used for Immunohistochemistry Analyze

	HMGB-1	MMP-9
Male	67	34
Female	33	16
Squamous carcinoma	41	20
Adenocarcinoma	52	27
Squamous adenocarcinoma	4	2
Large cell carcinoma	3	4
Period I-II	42	21
Period III-IV	58	26

RT-PCR and Western blot analyses of HMGB-1

Total RNA of tissues were isolated and reversed transcribed. HMGB-1 (NM-002128) was detected by RT-PCR using specific primers (F: ATATGGCAAAAGCGGACAAG, R: AGGCCAGGATGTTCTCCTTT). β-actin was considered to be control (F: CTCTGGCCGTACCACTGGC, R: GTGAAGCTGTAG CCGCGC). The PCR program included 95°C for 2 min, 40 cycles at 94°C for 20 s, 60°C for 20s and 72°C for 30s. RT-PCR products were documented with high resolution gel electrophoresis. The PCR was carried out by qPCR (ABI 2720, U.S.A), and relative expression of HMGB-1 was calculated by CT value. Besides, Western blot was carried out by HMGB-1 antibody (abcam 79823) (1:5000). Semi-quantitative gel image was analyzed by Imaging Systems GDS-8000 (UVP, U.S.A).

Immunohistochemistry

Samples were fixed in 4% paraformaldehyde overnight and decalcified with 0.1 M EDTA/PBS at room temperature before paraffin embedding and sectioning. Five-micrometer longitudinal sections were dewaxed in xylene followed by a graded series of ethanol washes (100% twice, 95% once, and 70% once) (Park et al., 2012). For immunohistochemistry, sections were incubated in 3% H2O2 for 15 min at room temperature, followed by antigen retrieval by incubation in 10 mM sodium citrate at 95 °C for 30 min and 0.1% Triton X-100 for 10 min, then the samples were blocked with 5% goat serum in PBS. Sections were incubated with primary antibody (HMGB-1, abcam 79823; MMP-9, abcam 38898) overnight at 4°C and washed (four times) with PBS, secondary antibody was applied according to manufacturers' recommendations. For detection, images were taken under microscope (Olympos BX51T-PHD-J11) using Image Pro Plus (Media Cybernetics). Expression intensity was calculated by A×B. A represents positive stained cells (0-1%=0, 1-25%=1, 25-50%=2, 50-75%=3, 75-100%=4). B represents intensity of positive stained cells (no staining=0, faint yellow=1, brown=2, dark brown=3). A×B>3 represents positive expression.

Statistical analyses

All data were expressed as mean±SD. Statistical analysis was treated by SPSS version 17.0 (SPSS Inc., Chicago, IL). Comparison between different groups was performed using t test (LSD-t). A p-value less than 0.05 was considered to be significantly different. Correlation analysis of HMGB-1 and MMP-9 was carried out by Pearson.

Results

The expression level of HMGB-1 in NSCLC

In order to observe the expression level of HMGB-1 in NSCLC, 30 NSCLC tissues were analyzed by both RT-PCR and Western blot. As a result, the average expression level of HMGB-1 in NSCLC patients was significantly higher than the normal lung tissues (p>0.05) (Figure 1).

Immunohistochemistry of HMGB-1 and MMP-9

HMGB-1 was analyzed by immunohistochemistry in

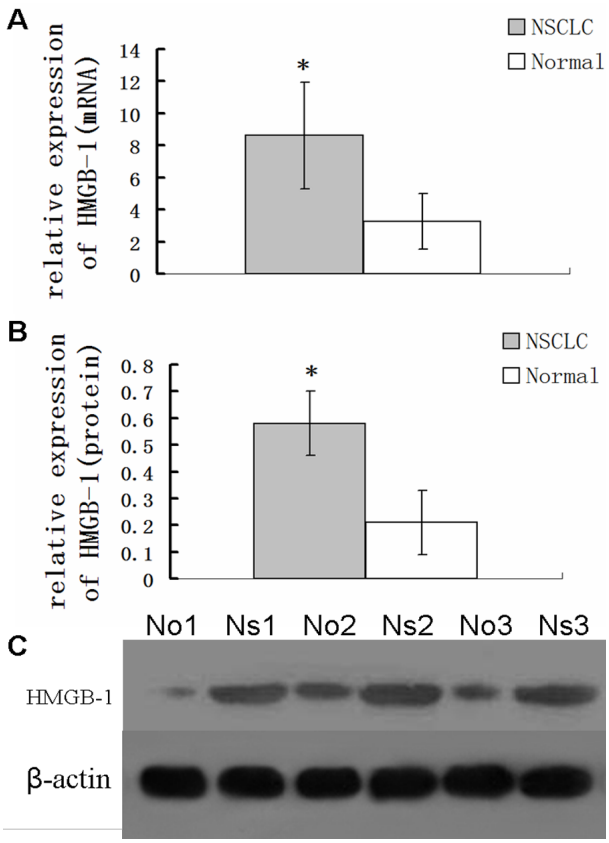

Figure 1. The Expression Level of HMGB-1. A: relative expression of HMGB-1 (RT-PCR, mRNA), N=30, β-actin was considered to be control. B: relative expression of HMGB-1 (Western blot, protein), N=30. *P<0.05 represent significantly different. C: Western blot analysis of three cases. No: normal tissues; Ns: NSCLC tissues. Case 1: male at age of 30, poorly differentiated adenocarcinoma combined with ipsilateral pulmonary metastasis and subcarinal lymphnode metastasis; Case 2: female at age of 55, poorly differentiated adenocarcinoma without metastasis; Case 3: male at age of 53, moderately differentiated squamous cell carcinoma combined with Ipsilateral hilar lymph node metastasis

100 NSCLC tissues and 30 normal tissues. As a result, there were 71 positive expression cases (positive rate 71%). In normal tissues, 10 cases exhibit positive (positive rate 33.3%). No difference was found among the various gender, age and pathological type of patients. However, patients in different stages showed different expression intensity. In 42I-II period cases, there were 24 cases exhibt positive expression (positive rate 51.7%). And in 58 III-IV period cases, the positive rate increased to 81.0% (47 positive expression cases). Figure 2A, B exhibit one of the immunohistochemistry results of the moderately differentiated adenocarcinoma (female at age of 59). Besides, we also analyzed the expression of MMP-9 in 50 NSCLC tissues and 30 normal tissues. As a result, the positive rate was 76% (38 positive expression cases) and 23.3% (7 positive expression cases) in NSCLC and normal tissues, respectively. Figure 2C, D represent a male case at age of 55 with adenocarcinoma.

Correlation between HMGB-1 and MMP-9

We speculate there maybe some relationship between HMGB-1 and MMP-9 because of the similar expression

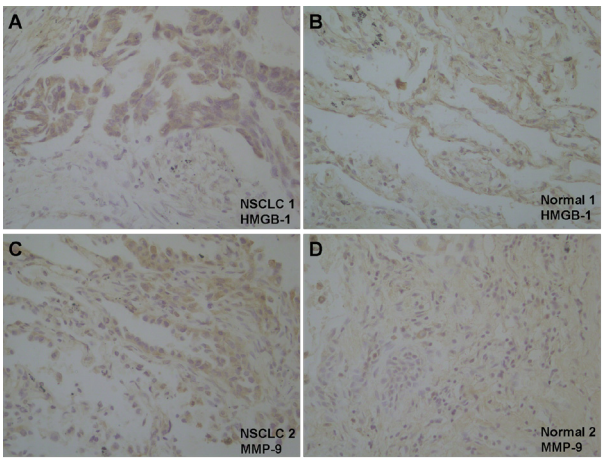

Figure 2. Immunohistochemistry of HMGB-1 and MMP-9. A: HMGB-1 in NSCLC tissues; B: HMGB-1 in normal tissues; C: MMP-9 in NSCLC tissues; D: MMP-9 in normal tissues. Case1: a female at age of 59 with moderately differentiated adenocarcinoma; Case2: a male at age of 55 with adenocarcinoma

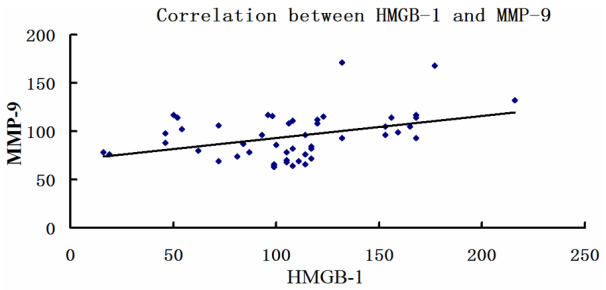

Figure 3. Correlation Between HMGB-1 and MMP-9 Analyzed by Pearson. Oblique line represents a significant correlation

level in NSCLC. Therefore, we performed Pearson analysis on HMGB-1 and MMP-9. As shown in Figure 3, a significant correlation was found in the expression of HMGB-1 and MMP-9.

Discussion

Nowadays, lung cancer especially NSCLC become one of the most common leading cause of death in the world. Most patients with lung cancer are in an advanced stage when at diagnosis, in which some appeared distant metastasis (Quint et al., 1996). Therefore, how to early diagnosis of lung cancer became the key point to improve the survival rate. In recent years, with the development of molecular biology, lung cancer indicators and targeted therapy have become a hot research topic. In this study, HMGB-1 was analyzed by RT-PCR and Western blot. Immunohistochemistry of HMGB-1 and MMP-9 was also carried out in various pathological types and clinical stage of NSCLC. As a result, HMGB-1 was significantly higher expressed in NSCLC patients, and it was associated with the disease stage. Besides, a positive correlation with HMGB-1 was found in the expression of MPP-9.

HMGB-1 was considered to be a cancer-promoting gene which was correlated with the occurrence and development of tumor. It could increase the cell ability of proliferation

migration, and metastasis. HMGB-1 was found to be highly expressed in many kinds of tumor, such as breast cancer, stomach cancer, colon cancer and colorectal cancer. In addition, the expression of HMGB-1 was usually related to tumor invasion and lymph node metastasis (LiuTsai et al., 2010). As reported, HMGB-1 was overexpressed in colon and colorectal cancer, and closely related to the depth of tumor invasion and the stage of lymph node metastasis (Luoand Kuniyasu, 2011); The mean value of serum HMGB-1 levels in patients with lung cancer was significantly higher than those in COPD patients, and healthy controls (Shang et al., 2009); In gastric carcinoma, the HMGB-1 acceptor RAGE appears to be closely associated with invasion and metastasis (Kuniyasu et al., 2002); HMGB-1 protein levels were significantly elevated in 90% of the carcinomas, and a strong correlation was exhibited between upregulation of the apoptosis repressing HMGB-1 and c-IAP2 proteins in the pathogenesis of colon carcinoma (Völp et al., 2006); Besides, neutralizing HMGB-1 was also found to be able to decrease the tumor incidence and size in a rat model of colorectal cancer (Maeda et al., 2007). In this study, the expression of HMGB-1 in NSCLC was significantly higher than normal lung tissues. Besides, it was found to be associated with the stage of lung cancer. Patients in III-IV period exhibit significantly higher positive rate when compared with I-II period cases. This indicates that HMGB-1 may be involved in the invasion and metastasis of lung cancer, and associated with the prognosis of tumors. High expression of HMGB-1 may affect the growth, invasion and metastasis of tumor by regulating some certain genes, such as tumor suppressor, DNA repair, recombination, cell adhesion, cell movement, cell invasion and angiogenesis regulating genes. These genes may exercise its function through specific HMGB-1 signaling pathways, such as RAGE, TLR2 and TLR4 (Taguchi et al., 2000; Takada et al., 2004; Wang et al., 2004; Dumitriu et al., 2005).

The expression of MMP-9 in NSCLC was also detected and the correlation with HMGB-1 was revealed in this study. Our results showed that the expression of MMP-9 in NSCLC was also significantly increased, and a positive correlation with HMGB-1 was found. MMP-9 may be involved in the occurrence and development mechanisms of NSCLC. As known, invasion and metastasis are the basic characteristics of tumor cells and important factors affecting the prognosis of patients. Basement membrane and extracellular matrix are natural barriers to hinder tumor invasion and metastasis. The integrity of basement membrane and extracellular matrix was necessary to avoid the tumor invasion and metastasis (Stetler-Stevenson et al., 1993). As reported, MMPs have been identified as important players in angiogenesis, growth and metastasis of tumors (Kleinand Bischoff, 2011). During the invasion of tumor, HMGB-1 could combined with ligand RAGE, then signaling pathways MAPK was activated, and lead to the activation of MMP-9. Finally, MMP-9 degrade the extracellular matrix and promoting tumor invasion and metastasis (Kuniyasu et al., 2005; Yang et al., 2005).

To sum up, HMGB-1 was highly expressed in NSCLC, which MPP-9 was positively correlated. HMGB-1 may be a useful clinical marker for evaluating the NSCLC progression and is of potential prognostic value.

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