Original paper

Expression of HMGB1 and NF-κB p65 and its significance in non-small cell lung cancer

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Aim of the study: To study high mobility group protein B1 (HMGB1) and nuclear transcription factor p65 (NF-κB p65) expression in non-small cell lung cancer and its significance.

Material and methods: 106 hospitalized patients with non-small cell lung cancer after thoracic surgery were enrolled; HMGB1 and p65 protein expression was detected by the immunohistochemical method. Semiquantitative expression of HMGB1 and NF-κB p65 was analyzed using Image Pro Plus (IPP) software and statistical analysis.

Results: The rate of HMGB1 positive expression in the non-small cell lung cancer protein B1 family was significantly higher than normal tissues ($P < 0.05$); p65 protein expression in the non-small cell lung carcinoma group was significantly higher than that of normal tissues ($P < 0.05$). HMGB1 and NF-κB p65 protein expression was significantly higher compared with the non-metastatic group ($P < 0.01$). HMGB1 and NF-κB p65 protein expression showed a positive correlation ($P < 0.05$).

Conclusions: HMGB1 and NF-κB p65 expression may be related to non-small cell lung cancer metastasis.

Key words: non-small cell lung cancer, high mobility group protein B1, nuclear transcription factor p65, transfer, immunohistochemistry.

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Introduction

Lung cancer is one of the most common malignancies in the world. In recent years, its incidence and mortality keep rising, and the survival rate is as poor as 12.5% [1–4]. Results of the study in the Polish population confirmed the efficacy of erlotinib in advanced NSCLC after failure of prior platinum-based chemotherapy [5]. High mobility group protein B1 (HMGB1), a non-histone chromosomal protein in eukaryotic cells, correlates highly with invasion and metastasis in multiple tumors. As revealed by previous research, tumor exogenous may be enhanced by HMGB1, which improves the production ratio of matrix metalloproteinases-9 (MMP9) and optimizes MMP9 gene activity, through activating p65 protein, one of the nuclear transcription factors, and adhering to the key site of the MMP9 gene [6–9]. The p53 gene and protein are significant in the elimination of impaired cells, through the path of apoptosis. In the mutation of the p53 gene, abnormal p53 protein is created. The biggest number of cells which accumulate protein p53 has been disclosed in the cells of adenocarcinoma whereas the smallest number of cells which accumulate protein p53 has been disclosed in multicellular types of cancer, and a smaller number of cells accumulating protein p53 has been disclosed in older patients [10].

With a sample including 106 non-small cell lung cancer (NSCLC) patients, we carried out a pathological study on content of p65 protein, the nuclear transcription factor, and HMGB1, to analyze their correlation with various clinical parameters, and discuss their effects on the enhanced exotrophy in NSCLC. Our results are presented here.

Material and methods

Clinical data

This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of the First Affiliated Hospital of Zhengzhou University. Written informed consent was obtained from all participants. This retrospective research included 106 NSCLC patients in hospitalized by our hospital from February 2009 to February 2011, 76 males and 30 females, aged between 32 and 80 years, average 60.2 y. All patients underwent thoracotomy, without neo-adjuvant chemotherapy or radiotherapy.

Immunohistochemistry

Routine paraffin sectioning, dewaxing, and hydration using 3% hydrogen peroxide were performed to remove endogenous peroxidase. Microwave antigen was retrieved and blocked with fetal calf serum for 2 h. About 50 μl (1 : 25) of goat polyclonal anti-human HMGB1 and NF-κB p65 were added, and the mixture was incubated at 4°C overnight. About 50 μl of biotinylated goat

anti-rabbit IgG secondary antibody working solution was added, and the mixture was incubated at 37°C for 30 min, followed by diaminobenzidine coloration. The sample was dyed with hematoxylin, separated using ethanol and hydrochloric acid, saturated with lithium carbonate until the color returned to blue, and then dehydrated with gradient alcohol as well as xylene. Mounting with neutral resin followed. The negative control used was PBS in place of the primary antibody.

In immunohistochemistry (IMC) SP, the sample appearing yellow or brownish yellow was considered as positive. Quantity and intensity of the stained cells were evaluated by the staining in each of ten fields in 400-fold magnification for 100 cells per observation. A sample was scored 0 for positive cells below 10%, 1 for 11–25%, 2 for 26–50%, 3 for 51–75%, and 4 for higher than 75%. Microscopic staining intensity for the positively stained cells was scored 1 for the yellow, 2 for the brownish yellow, and 3 for the brown. The final observation result, a product of the percentage of the positive cells out of those under observation multiplied by the staining intensity, was considered as negative for the product 0, which means no less than 10% positive cells were found, weak positive for 1–4, which means the average number of positive cells was less than 25%, moderate positive for 5–8, which means the average number of positive cells was less than 50%, and strong positive for 9–12, which means the average number of positive cells was more than 50%, wherein the former two were regarded as low expression in HMGB1, and the latter two as high expression.

Statistical analysis

Semiquantitative expression of NF-κB p65 and HMGB1 was analyzed by the score. Only 0 was negative, while others were all considered as positive. χ^2 test was conducted to test the relevance of the expression of NF-κB p65 (or HMGB1) and the tumor size, histomorphological types, tumor mesenchyme, differentiation degree and lymph node metastasis. Meanwhile, the relation between NF-κB p65 and HMGB1 was also evaluated with χ^2. $P < 0.05$ was considered as a significant difference.

Results

Clinical data

The clinical data are shown in Table 1. The patients included 65 squamous carcinomas, 25 adenocarcinomas, 16 adenosquamous carcinomas, 64 with node metastasis and 42 without, 36 in stage I, 22 in stage II and 48 in stage III, 50 poorly differentiated, 39 moderately differentiated and 17 well differentiated. Paracarcinomatous tissue beyond 5 cm from the tumor margin was collected from 32 patients during the operation as the control.

Expression of HMGB1 and p65

High expression of HMGB1 was found in both cytoplasm and nucleus of both tumor cells and interstitial inflammatory cells. Expression of the nuclear transcription factor p65 was noted in cytoplasm and nucleus of tumor cells with the intensity from faint yellow to brownish yellow, but generally null in the interstitial cells. Positive expression of either HMGB1 ($p < 0.05$) or p65 ($p < 0.01$) was higher significantly in NSCLC tissue than in the control, the paracarcinomatous tissue. The expressed quantity of either HMGB1 or p65 was significantly higher in patients with node metastasis than in those without it ($p < 0.01$).

Correlation analysis

Expression of p65 was observed in 36 (70.59%) out of 51 NSCLC tumor tissues high in HMGB1 expression, and in only 12 (21.81%) out of 55 low in HMGB1, and there was a relation between NF-κB p65 and HMGB1 ($p < 0.05$).

Discussion

Prognosis highly correlates with metastasis in lung cancer patients. HMGB1 was first discovered to be an omnipresent DNA-binding protein, which regulates the genesis of transcription complex and therefore participates in the transcription, replication and repair of DNA and cellular mobility, through inducing the transfiguration of chromosomes and DNA [11–15]. Secreted by macrophages, monocytes or damaged necrotic cells, HMGB1 induces a chemotactic response, and therefore participates in metastasis of tumor cells [16–18]. HMGB1 in 95D human lung cancer cells, HMGB1 alone or acting synergistically with CpG ODN could enhance the progression of 95D cells, which would promote the progression of lung cancer [19]. Our research revealed its high expression in tumor cells and interstitial cells, and significantly higher expression in tumors with node metastasis than in those without it, which seems similar to the results of previous research on cervix cancer and colon cancer, implying a correlation between positive HMGB1 expression and node metastasis in NSCLC.

P65 protein, a transcription factor first separated from materials contained in the nucleus of mature immune cells, is a molecule involved in cellular signal transduction, which influences the activity of transcription factors through various mechanisms, by all means, in all ways, and therefore intensifies or attenuates cellular functions or activities in different stages of the life cycle [20, 21]. Skeletal metastases are a frequent complication of lung cancer, and p65 was one of the signal proteins involved in the skeletal complications of cancer metastases [22]. Therefore, p65 became one of the foci in research on mechanisms involved in oncogenesis. Fujioka *et al.* revealed a positive correlation between the transcription factor p65 and tumor metastasis. It has been confirmed in pulmonary cancer that RelA/p65 is necessary to link smoke-induced inflammation and has a role in the activation of Wnt/β-catenin signaling in tumor cells [23]. In our research, expression of p65 was found positive in cytoplasm and nucleus of tumor cells but null generally in the interstitial cells. Furthermore, significantly higher expression of both HMGB1 and p65 was observed in tumors with node metastasis than in those without it, implying a certain close correlation between tumor metastasis and HMGB1.

This research also suggests that there is a positive correlation between HMGB1 and the transcription factor p65 in NSCLC. Some other research found that HMGB1 and p65 were important factors in melanoma progression [24]. Therefore,

Table 1. Relationship between HMGB1, NF-κB p65 expression and the clinic pathological features of NSCLC

Clinic pathological parameters	*n*	NF-κB p65 positive expression [cases (%)]	x^2 value	*P* value	HMGB1 positive expression [cases (%)]	x^2 value	*P* value
Tumor size							
≤ 3 cm	48	21 (43.75)	0.007	0.929	23 (47.92)		
> 3 cm	58	26 (44.83)	0.027	0.849	28 (48.28)		
Histomorphological types							
Squamous cancer	65	22 (33.80)			25 (38.46)		
Adenocarcinoma	25	14 (56.00)	9.386	0.009	16 (64.00)	6.697	0.035
Adenosquamous carcinoma	16	12 (75.00)			11 (69.00)		
Tumor mesenchyme							
Squamous carcinoma	65	0			51 (78.46)	8.699	0.003
Adenocarcinoma	25	0			10 (40.00)		
Differentiation degree							
Low	50	29 (58.00)			31 (62.00)		
Median	39	13 (33.03)	7.084	0.028	16 (41.03)	7.647	0.021
High	17	4 (23.53)			4 (23.53)		
Lymph node metastasis							
Yes	64	38 (59.38)			42 (65.63)		
No	42	9 (21.43)	12.438	0.000	9 (21.43)	17.258	0.000

Note: HMGB1: nuclear transcription factor protein B1; NF-κB p65: Nuclear transcription factor p65 protein

NF-κB p65

Tumor size	Positive	Negative	Total
< 3 cm	21 (a)	27 (b)	48 (a + b)
≥ 3 cm	26 (c)	32 (d)	58 (c + d)
Total	47 (a + c)	59 (b + d)	106 (*n*)

$n = 106 > 40$
$T_{11} = [(a + b) \times (a + c)]/n = 48 \times 47/106 = 21.28 > 5;$
$T_{12} = [(a + b) \times (b + d)]/n = 48 \times 59/106 = 26.72 > 5;$
$T_{21} = [(c + d) \times (a + c)]/n = 58 \times 47/106 = 25.72 > 5;$
$T_{22} = [(c + d) \times (b + d)]/n = 58 \times 59/106 = 32.28 > 5;$
$x^2 = (a - T_{11})^2/T_{11} + (b - T_{12})^2/T_{12} + (c - T_{21})^2/T_{21} + (d - T_{22})^2/T_{22} = 0.0012;$
$v = 1;$
$P \approx 0.7$

HMGB1

Tumor size	Positive	Negative	Total
≥ 3 cm	23 (a)	25 (b)	48 (a + b)
< 3 cm	28 (c)	30 (d)	58 (c + d)
Total	51 (a + c)	55 (b + d)	106 (*n*)

$n = 106 > 40$
$T_{11} = [(a + b) \times (a + c)]/n = 48 \times 51/106 = 23.09 > 5;$
$T_{12} = [(a + b) \times (b + d)]/n = 24.91 > 5;$
$T_{21} = [(c + d) \times (a + c)]/n = 27.91 > 5;$
$T_{22} = [(c + d) \times (b + d)]/n = 30.09 > 5;$
$x^2 = (a - T_{11})^2/T_{11} + (b - T_{12})^2/T_{12} + (c - T_{21})^2/T_{21} + (d - T_{22})^2/T_{22} = 0.0012;$
$v = 1;$
$P \approx 0.7$

NF-κB p65

Histomorphological types	Positive	Negative	Total
Squamous cancer	22	43	65
Adenocarcinoma	14	11	25
Adenosquamous carcinoma	12	4	16
Total	48	58	106

$n = 106;$
$T_{11} = 65 \times 48/106 = 29.43 > 5;$
$T_{12} = 65 \times 58/106 = 35.57 > 5;$
$T_{21} = 25 \times 48/106 = 11.32 > 5;$
$T_{22} = 25 \times 58/106 = 13.68 > 5;$
$T_{31} = 16 \times 48/106 = 7.25 > 5;$
$T_{32} = 16 \times 58/106 = 8.75 > 5;$
$x^2 = 10^6[22^2/(65 \times 48) + 43^2/(65 \times 58) + 14^2/(48 \times 25) + 11^2/(58 \times 25) + 12^2/(48 \times 16) + 4^2/(58 \times 16) - 1] = 10.2928;$
$0.05 < p < 0.01$

Table 1. Cont.

HMGB1

Histomorphological types	Positive	Negative	Total
Squamous cancer	25	40	65
Adenocarcinoma	16	9	25
Adenosquamous carcinoma	11	5	16
Total	52	54	106

$n = 106;$
$T_{11} = 65 \times 52/106 = 31.89 > 5;$
$T_{12} = 65 \times 54/106 = 33.11 > 5;$
$T_{21} = 25 \times 52/106 = 12.26 > 5;$
$T_{22} = 25 \times 54/106 = 12.74 > 5;$
$T_{31} = 16 \times 52/106 = 7.85 > 5;$
$T_{32} = 16 \times 54/106 = 8.15 > 5;$
$x^2 = 106 [25^2/(65 \times 52) + 40^2/(65 \times 54) + 16^2/(52 \times 25) + 9^2/(54 \times 25) + 11^2/(52 \times 16) + 5^2/(54 \times 16) - 1] = 35.24;$
$= (2 - 1) \times (3 - 1) = 2;$
$P < 0.005$

NF-κB p65

Differentiation degree	Positive	Negative	Total
Low	29	21	50
Median	13	26	39
High	4	13	17
Total	46	60	106

$n = 106;$
$T_{11} = 50 \times 46/106 = 21.70 > 5;$
$T_{12} = 50 \times 60/106 = 28.30 > 5;$
$T_{21} = 39 \times 46/106 = 16.92 > 5;$
$T_{22} = 39 \times 60/106 = 17.66 > 5;$
$T_{31} = 17 \times 46/106 = 7.38 > 5;$
$T_{32} = 17 \times 60/106 = 9.62 > 5;$
$x^2 = 106 [29^2/(50 \times 46) + 21^2/(50 \times 60) + 13^2/(39 \times 46) + 26^2/(39 \times 60) + 4^2/(17 \times 46) + 13^2/(17 \times 60) - 1] = 8.6746;$
$v = (2 - 1) \times (3 - 1) = 2;$
$P < 0.025$

HMGB1

Differentiation degree	Positive	Negative	Total
Low	31	19	50
Median	16	23	39
High	4	13	17
Total	51	55	106

$n = 106;$
$T_{11} = 50 \times 51/106 = 24.06 > 5;$
$T_{12} = 50 \times 55/106 = 25.94 > 5;$
$T_{21} = 39 \times 51/106 = 18.76 > 5;$
$T_{22} = 39 \times 55/106 = 20.24 > 5;$
$T_{31} = 17 \times 51/106 = 8.18 > 5;$
$T_{32} = 17 \times 55/106 = 8.82 > 5;$
$x^2 = 106 [31^2/(50 \times 51) + 19^2/(50 \times 55) + 16^2/(39 \times 51) + 23^2/(39 \times 55) + 4^2/(17 \times 51) + 13^2/(17 \times 55) - 1] = 8.7615;$
$V = (2 - 1) \times (3 - 1) = 2;$
$P < 0.025$

NF-κB p65

Lymph node metastasis	Positive	Negative	Total
Yes	38 (a)	26 (b)	64 (a + b)
No	9 (c)	33 (d)	42 (c + d)
Total	47 (a + c)	59 (b + d)	106 (*n*)

$n = 106 > 40$
$T_{11} = [(a + b) \times (a + c)]/n = 64 \times 47/106 = 29.38 > 5;$
$T_{12} = [(a + b) \times (b + d)]/n = 64 \times 59/106 = 35.62 > 5;$
$T_{21} = [(c + d) \times (a + c)]/n = 42 \times 47/106 = 18.62 > 5;$
$T_{22} = [(c + d) \times (b + d)]/n = 42 \times 59/106 = 23.38 > 5;$
$x^2 = (a - T_{11})^2/T_{11} + (b - T_{12})^2/T_{12} + (c - T_{21})^2/T_{21} + (d - T_{22})^2/T_{22} = 13.9298;$
$v = 1;$
$P < 0.005.$

Table 1. Cont

HMGB1			
Lymph node metastasis	**Positive**	**Negative**	**Total**
Yes	42 (a)	22 (b)	64 (a + b)
No	9 (c)	33 (d)	42 (c + d)
Total	51 (a + c)	55 (b + d)	106 (*n*)

n = 106 > 40
$T_{11} = [(a + b) \times (a + c)]/n = 64 \times 51/106 = 30.79 > 5;$
$T_{12} = [(a + b) \times (b + d)]/n = 64 \times 55/106 = 33.21 > 5;$
$T_{21} = [(c + d) \times (a + c)]/n = 42 \times 51/106 = 20.21 > 5;$
$T_{22} = [(c + d) \times (b + d)]/n = 42 \times 55/106 = 21.79 > 5;$
$x^2 = (a - T_{11})^2/T_{11} + (b - T_{12})^2/T_{12} + (c - T_{21})^2/T_{21} + (d - T_{22})^2/T_{22} = 19.8502;$
v = 1;
P < 0.005

Relation between p65 and HMGB1			
p65	HMGB1(+)	HMGB1(–)	Total
p65(+)	36 (a)	12 (b)	48 (a + b)
p65(–)	15 (c)	43 (d)	58 (c + d)
Total	51 (a + c)	55 (b + d)	106 (*n*)

n = 106 > 40
$T_{11} = [(a + b) \times (a + c)]/n = 48 \times 51/106 = 23.09 > 5;$
$T_{12} = [(a + b) \times (b + d)]/n = 24.91 > 5;$
$T_{21} = [(c + d) \times (a + c)]/n = 27.91 > 5;$
$T_{22} = [(c + d) \times (b + d)]/n = 30.09 > 5;$
$x^2 = (a - T_{11})^2/T_{11} + (b - T_{12})^2/T_{12} + (c - T_{21})^2/T_{21} + (d - T_{22})^2/T_{22} = 25.4196;$
v = 1;
P < 0.005

we speculate that these two molecules might not only be adopted as markers in a joint detection to help evaluate the prognosis of NSCLC patients, but also regarded as potential targets under investigation in cancer therapy.

The authors declare no conflict of interest.

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