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Clinical signification of high-mobility group box 1 protein (HMGB1) expression in infiltrating ductal carcinoma breast tissue*

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Abstract ***Objective:*** Exploring the clinical signification of high-mobility group box 1 protein (HMGB1) expression in infiltrating ductal carcinoma (IDC) breast tissue. ***Methods:*** The expression of HMGB1 protein in IDC breast tissue was detected by immunohistochemistry, and the relations among size of tumour, lymph node metastasis, clinical staging, estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER-2) were also analyzed. ***Results:*** Forty-six cases out of 60 cases of IDC breast tissue showed positive or strong positive HMGB1 expression (76.67%), statistical significance was observed between HMGB1 expression with clinical staging ($P < 0.01$), lymph node metastasis ($P < 0.01$), breast cancer ER ($P < 0.05$) and HER-2 ($P < 0.05$), however same conclusion can not be drawn between HMGB1 with either size of tumour or PR expression ($P > 0.05$) in IDC breast tissue. Spearman analysis showed negative correlation between HMGB1 expression and ER, and positive correlation between HMGB1 expression and clinical staging, lymph node metastasis together with HER-2. ***Conclusion:*** It's promising that HMGB1 expression in IDC tissue can be one of biological indicators of poor prognosis.

Key words infiltrating ductal carcinoma (IDC); high-mobility group box 1 protein (HMGB1); clinical staging; lymph node; estrogen receptor (ER); human epidermal growth factor receptor 2 (HER-2)

Breast cancer is the most frequent female malignant tumour. Although huge progresses have been made in cancer treatment field within the recent 20 years and effectiveness of treatment has been greatly improved [1] with the help of advances in biomarker research, recognition of breast cancer heterogeneity and various clinical manifestations of different subtype together with targeted therapy, breast cancer still take places in 29% of de novo female malignant tumour, ranking first [2]. According to recent researches, invasion and metastasis are not only the inherent and significant biological characteristics of breast cancers, but also the main reasons of breast cancer treatment failure and causes of death as well. FRA-1 plays an important role in breast cancer metastasis, invasion and maintaining the malignant phenotype [3]. Other researches indicated that different response rate of neoadjuvant chemotherapy to different molecular subtypes in regional advanced breast cancer such as ER, PR and HER-2 [4]. As shown in our previous research, miR-200c can inhibit invasion and metastasis of breast cancer cell by regulating high-mobility group box 1 protein (HMGB1) expression [5]. Our study detected the HMGB1 protein expression in infiltrating ductal carcinoma (IDC) breast tissue and discussed its clinical signification in breast cancer tissue, and the correlations among size of tumour, lymph node metastasis, clinical staging, estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER-2).

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Materials and methods

Clinical data

A total of 60 patients diagnosed with IDC through core needle biopsy (CNB) or surgical resection were involved from March 2012 to October 2013. All patients were female, aged from 20 to 72 years old, with a median age of 56.2 years. According to the clinical staging and AJCC TNM classification (2011) for breast cancer, among all primary tumour (T), 14 cases were T1, 25 cases were T2, 16 cases were T3 and 5 cases were T4. Regional lymph nodes metastasis (N): N = 0 in 16 cases, N < 10 in 26 cases and 18 cases of N ≥ 10. Distant metastasis (M): 54 cases of M0 and 6 cases of M1. Clinical staging: 10 cases of stage I, 23 cases of stage III, 21 cases of stage III and 6 cases of stage IV. None of patients has been treated with chemotherapy or radiotherapy before surgery.

Materials

Immunohistochemical SP kit, DAB kit, primary and secondary antibodies of ER, PR and HER-2 were all purchased from Zhongshan Biotechnology Ltd., Beijing (China). Anti-human HMGB1 antibody was purchased from Sigma Ltd., USA. PathVysion HER-2 DNA probe kit was purchased from Vysis Inc., USA.

Immunohistochemistry (IHC)

Samples *in vitro* should be placed in fixative (fixative volume / sample volume = 10) as soon as possible within one hour. The times of separation and placing into fixative were recorded. Tissue specimen were cut into 5 mm cubes and fixed into 10% neutral buffered formalin (NBF) for 6–72 h. ER, PR and HER-2 were prepared with standard procedure: dehydrate with ethanol, embed with paraffin, serial section by each 5 μm and then conduct HE (hematoxylineosin staining) and IHC staining respectively. IHC was performed with SP (streptavidin-peroxidase) three-step-method: paraffin-embedded sections were deparaffinaged, microwave antigen retrieval with citrate buffer, endogenous enzyme was inactivated with 3% hydrogen peroxide for 40 min, then washed with PBS and blocked with goat serum for 30 min. HMGB1 staining: anti-human HMGB1 antibody (1:100) was used as the primary antibody, stored overnight at 4 ℃, then added biotin as secondary antibody at 37 ℃ for 30 min, all slides were washed with PBS after each antibody application; incubated with horseradish-streptavidin peroxidase for 20 min, stained with DAB, counterstain with hematoxylin, dehydrated, and mounted. All sections were observed under microscope.

Fluorescence in situ hybridization (FISH)

All samples were blocked in 10% NBF for 24–28 h, embedded paraffin sections were cut as 5-μm-thick serial sections. Blocking samples in 0.2 NHCL for 20 min, washing with pure water and 2 × SSC buffer solution. Putting samples in 1M NaSCN at 80 ℃ for 30 min, washing, then the slices were put in protease solution at 37 ℃ for 10 min (250 mg/500 mL, 0.01 NHCL). After washing with pure water, the slices were dried at 50 ℃ for 2–5 min and denatured at 72 ℃ for 5 min. Treating with 75%, 85% and 100% alcohol for 1 min respectively and allowing to dry in air; adding 10 μL probe mixture at target region of tumour tissues, covering with 18 × 18 mm coverslip and sealing with wax glue. Storing it within in situ hybridization apparatus overnight at 37 ℃. Float coverslip within 2 × SSC, 0.3% NP-40 at washing liquor. After 2 min at 72 ℃, placed it in the dark at room temperature, added 10 μL DAPI color reagent at target region of tumour tissue, sealed with coverslip and observed under fluorescence microscope.

Control group establishment

Positive and negative control groups were established in our study. All pathological specimen were prepared at the same condition. Positive staining section was used as positive external control, while PBS instead of primary antibody as negative external control. Positive internal control was normal luminal epithelial cell, and negative control was myoepithelial cell and mesenchymal cell of normal breast tissue.

Result determination

The results were evaluated according to the guidelines of American Society of Clinical Oncology (ASCO) [6, 7]. Tumor types and stages were determined by two experienced pathologists. Yellow or brown granule in cells was regarded as positive expression. A comprehensive assessment was carried out including the percentage of positive cells in each section and the degree of positive expression (weak, moderate, high).

ER, PR determination standard

The positive expressions of ER and PR were all distributed in nucleus, or both the nucleus and cytoplasm stained. The degree of positive expression was graded as follows: negative, < 1% of cells stained; positive, > 1% of cells stained, with the positive percentage grading as 10%, 20%, 30%, etc. Staining magnitudes (expression level) were divided into three different degrees (+, ++, +++,). The percentage and expression levels of tumour cells were included in result interpretation, such as 30% +++ or 50% ++.

HER-2 determination standard

The positive expression of HER-2 were mainly distributed in cytoplasm and on membrane, especially on membrane. HER-2 negative (+/0), weak or incomplete membrane staining at any percentage of infiltrating carcinoma cells or barely but completed membrane staining at < 10%

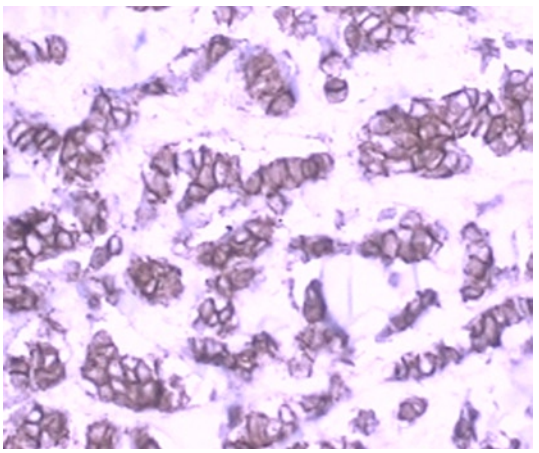

Fig. 1 Positive expression of HMGB1, 50% (++) (SP × 400)

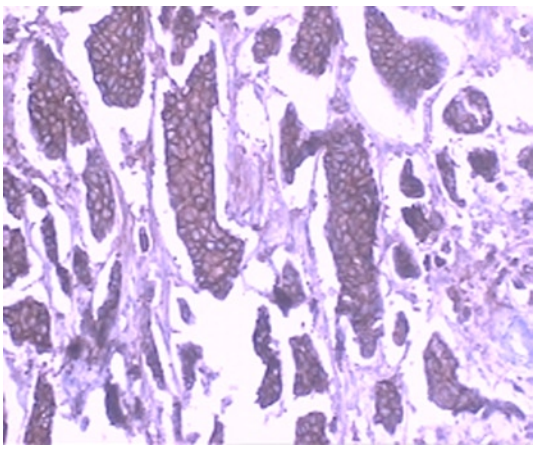

Fig. 2 Positive expression of HMGB1, 80% (+++) (SP × 400)

infiltrating carcinoma cells, or no staining at all; HER-2 expression uncertain (++), > 10% infiltrating carcinoma cells appear weak to moderate staining, complete but non-uniform distributed brownish yellow membrane staining or < 30% infiltrating carcinoma cells appear strong and complete reddish brown membrane staining; HER-2 positive (+++), > 30% infiltrating carcinoma cells appear strong and complete membrane reddish brown staining. A further FISH test is required if HER-2 expression (++): tumour cell nucleus shows blue fluorescence signal, the centromere of chromosome 17 shows green fluorescence signal, HER-2 gene shows red fluorescence signal. Calculation the ratio of number of red HER-2 gene fluorescence signal to green fluorescence signal in chromosome 17, Her-2 positive can be concluded if ratio > 2.2 while opposite conclusion can be concluded if < 1.8; If between 1.8 to 2.2, conclusion should be drawn combined with immunohistochemical result.

HMGB1 determination standard

HMGB1 positive distributed in nucleus and cytoplasm, mainly in nucleus. The scoring criteria was as follows: negative, no staining or positive cell < 10%; positive, positive cell > 10%, with the positive percentage grading as 10%, 20%, 30%, etc. Staining magnitudes (expression level) were divided into three different degrees (+, ++, +++,). The percentage and expression levels of tumour cells were included in result interpretation, such as 50% ++ or 80% +++ (Fig. 1 and 2).

Statistical analysis

Differences between two groups were analyzed by χ^2 test using SPSS 13.0 software. A P value < 0.05 from two-side tests was considered statistically significant. A further analysis was conducted with Spearman correlation analysis, r < 0.3 was considered as slightly correlated, 0.3 < r < 0.5 was considered as moderate correlated, 0.5 < r < 1 was considered as highly correlated.

Results

Out of 60 subjects with IDC, HMGB1 positive or strongly positive expression occurred in 46 IDC subjects (46/60, 76.67%). HMGB1 expression in stages III and IV were significantly higher than in stages I and II. The HMGB1 expression was significantly higher in lymph nodes metastasis tissues than those of no lymph nodes metastasis (P < 0.01). There was significant difference between HMGB1 expression and ER, HER-2 expression (P < 0.05), while no significant difference was observed between HMGB1 expression and tumor size, PR expression (P > 0.05; Table 1). Spearman correlation analysis showed negative correlation between HMGB1 and ER, while positive correlation between clinical staging, lymph metastasis and HER-2 expression (Table 2).

Discussion

HMGB1 is among the most important chromatin proteins, which can enhance the transcriptional activity of proteins such as NF-kB, p53, etc. In the nucleus HMGB1 interacts with nucleosomes, transcription factors, and histones. This nuclear protein organizes the DNA and regulates transcription [8]. HMGB1 is an intracellular protein that can translocate to the nucleus where it binds DNA and regulates gene expression. It can also be released from cells, in which extracellular form it can bind the receptor for advanced glycan endproducts (RAGE). The relations between HMGB1 and tumour invasion, migration and metastasis are discovered in recent years. High expression HMGB1 can activate HMGB1-RAGE signal pathway and induce tumour invasion and metastasis [9]. Ectopic expression of HMGB1 can induce apoptosis by inhibiting BTB. It can also reduce the expression of Bax and p53, enhance expression of Bcl-xL, Bcl-2, cyclin D1

Table 1 Expression of HMGB1 in IDC breast tissue and pathologic comparison between groups (χ^2 test)

Items	*n*	HMGB1 protein expression				χ^2	*P*
		+		–			
		n	%	*n*	%		
Tumour size (cm)							
≤ 2	14	10	71.43	4	28.57	0.280	0.597
> 2	46	36	78.26	10	21.74		
Clinical stage							
I or II	33	21	63.64	12	36.36	6.960	0.008**
III or IV	27	25	92.59	2	7.41		
Lymph nodes metastasis							
No	16	7	43.75	9	56.25	13.215	0.000**
Yes	44	39	88.64	5	11.36		
ER							
Negative	25	23	92.00	2	8.00	5.633	0.018*
Positive	35	23	65.71	12	34.29		
PR							
Negative	21	18	85.71	3	14.29	1.478	0.224
Positive	39	28	71.79	11	28.21		
HER-2							
Negative	25	15	60.00	10	40.00	6.655	0.010*
Positive	35	31	88.57	4	11.43		

* $P < 0.05$, ** $P < 0.01$

Table 2 Spearman correlation coefficients between groups of HMGB1 in IDC breast tissue

HMGB1 protein expression	Clinical staging (*n*)		Lymph nodes metastasis (*n*)		ER (*n*)		HER-2 (*n*)	
	I or II	III or IV	No	Yes	Negative	Positive	Negative	Positive
Negative	12	2	9	5	2	12	10	4
Positive	21	25	7	39	23	23	15	31
r	0.341		0.469		–0.230		0.333	

$r < 0.3$ is considered as slightly correlated, $0.3 < r < 0.5$ is considered as moderate correlated, $0.5 < r < 1$ is considered as highly correlated

and NF-κB, and regulate multiple mechanisms to involve and affect tumour progress [10]. HMGB1 has the function of transmitting infinite self-replication signal, inhibiting tumour cell apoptosis, increasing tumour vasculature neovascularization, and promoting tumour growth, invasion and metastasis [11]. During radiotherapy, treatment can be improved with cancer biology immunoreaction by activating effect of HMGB1 protein thus induce CTL effect [12]. It is proven by researches that though ubiquitin proteasome system, ER positive MCF-7 breast cancer cell apoptosis is along with releasing of HMGB1, which indicates that HMGB1 can be treatment marker of ER positive breast cancer [13]. Another researches shows that, as HMGB1 being a marker of apoptosis, soluble HMGB1 in plasma are even more sensitive than CA 15-3 or CEA, which indicates that HMGB1 can be a marker of immunogenic cell death are valuable for the diagnosis of MBC and early estimation of response to neoadjuvant therapy in LBC patients [14, 15]. In this research, HMGB1 expression in stages III and IV were significantly higher than in stages I and II. The HMGB1 expression was significantly higher in lymph nodes metastasis tissues than those of no lymph nodes metastasis. There was significant difference between HMGB1 expression and ER, HER-2 expression. There was negative correlation between HMGB1 and ER, while positive correlation exists between HMGB1 and HER-2. Because of ER correlating with favorable prognosis, while HER-2 relating to the opposite, we consider that HMGB1 has a positively correlation with clinical staging and lymph nodes metastasis. This results further demonstrates that HMGB1 can be one of the biomarkers of unfavourable prognosis. Our research reveals that there is certain relation between HMGB1 and the progression, invasion, metastasis of IDC. It is promising that HMGB1 will become a new target of prognosis, and treatment of breast cancer.

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