Immunohistochemical expression and significance of NM23 suppressor protein in primary gastric adenocarcinoma

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Abstract

NM 23 protein was originally identified as a metastasis suppressor protein. The expression of NM23 has been correlated with tumour metastatic potential in various human carcinoma, mostly in ductal breast and colorectal carcinomas. Evidence for their expression in gastric cancer is rather contradictory, both for protein expression status and prognostic value. This study was done to analyze the immunohistochemical expression of NM23 in gastric carcinoma, and correlation of the degree of staining with clinicopathological parameters was investigated.

In a retrospective immunohistochemical study specimens obtained from 56 gastric cancer patients who had undergone gastrectomy with perigastric lymphadenectomy were analysed, in correlation with classical clinical-pathological parameters of tumours, WHO-, Lauren-, Goseki-, and Ming- classification. NM 23 gene expression was compared in gastric adenocarcinoma and tumour-adjacent non-neoplastic gastric mucosa. A semiquantitative immunostaining evaluation (score 0-3) was used, counting the percentage of stained cells. Statistical analysis was performed using Kolmogorov-Smirnov test, and Spearman rank correlation test.

The investigated group consisted of 40 males and 16 females (2.5:1) with a mean age of 63 years (range: 48-81 years). The percentage of positive expression of NM23 (score 3) were in 30 (53.5%) specimens in non-neoplastic mucosa in adjacent gastric carcinoma, and negative (score 0-2) in all 56 (100%) specimens of gastric adenocarcinoma. NM23 expression was higher in non-neoplastic mucosa than in adjacent gastric adenocarcinoma tissue (p<0.0001). NM23 protein expression did not correlate with gender (p=0.115), tumour size (p=0.844), tumour grade (p=0.172), lymphovascular invasion (p=0.606), lymph node metastases (p=0.311), Lauren classification (p=0.426), Goseki classification (p=0.458) and Ming classification (p=0.212).

Our series did not show a significant correlation between NM23 expression and analysed clinico-pathological variables, but these results suggest that protein NM23 may have a role in gastric carcinoma pathogenesis.

KEY WORDS: gastric cancer, NM23, immunohistochemistry

INTRODUCTION

The gastric cancer is one of the most common cancer in the Western countries, with a persistently rising incidence. It is the second in cancer-related mortallity next to lung cancer. Metastasis is the main cause of death in this group, leading to locoregional or distant recurrence in late-stage tumours. The most important prognostic factors is UICC TNM stage determined by the depth of invasion, the involvement of the perigastric lymph nodes, and distant metastasis. It is essential to predict the risk of recurrence in order to minimize adverse effects and maximize the therapeutic effect in the treatment of cancer patients. There is a need for new prognostic and predictive factors other than the TNM stage, because the prognosis varies among patients of the same stage. Activation or inactivation of multiple genes is involved in the various steps of tumour progression. The molecular basis of the metastatic disease is not known. The development and progression of gastric cancer are results of multiple genetic alterations, like in many others cancers. Recently published studies revealed potential function of oncogenes and tumour suppressor genes, growth factors and receptors, cell adhesion molecules, proteolytic molecules and angiogenic factors, in the prognosis and prediction for gastric cancer [1]. Several molecular factors are studied as prognostic and predictive factors for gastric cancer. Biological markers - p53, PCNA, HER-2, known to be indicators of prognosis in gastric cancer [2]. The non metastatic NM23 gene was initially identified as

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Submitted: 29 June 2012 / Accepted: 6 March 2013

a putative metastasis suppressor gene on the basis of its reduced expression in certain highly metastatic cell lines and tumours [3]. The gene is located on chromosome locus 17q21, which encodes an 18.5 kDa protein containing 166 amino acid residues with nucleoside diphosphate kinase, histidine kinase and serine autophosphorylation activities [4]. In humans, there are 10 genes belonging to the NM23 gene family (also known as NME genes), of which the two most abundantly expressed are NM23-H1 (NME1) and NM23-H2 (NME2) [5]. The NM23 genes appear to play a critical role in cellular proliferation, differentiation, oncogenesis, and tumour metastasis [6]. A recent study delineated that NM23-H1 is critical for control of cell-cell adhesion and cell migration at early stages of the invasive program in epithelial cancers, orchestrating a barrier against conversion of in situ carcinoma into invasive malignancy [5]. Low NM23-H1 expression disrupted cell-cell adhesion and promoted cellular scattering, motility, and extracellular matrix invasion by upregulating several matrix metalloproteinases [5]. It is critical at early stages of the invasive cancers. Low NM23-H2 expression was ineffective at tumour invasion [5]. Up to now, it is clearly established that NM23-H1 is a critical regulator of signalling networks involved in cancer cell adhesion and local invasion in primary tumours [7]. Fan et al. suggested that NM23-H1 may be associated in DNA repair [8]. Mutation in NM23 genes are rare in cancer, and NM23 may be a family of cancer genes that become dysregulated through expression changes at protein level. Youssef et al. suggest that the role NM23 in oncogenesis and tumour metastasis could be related to its nucleoside diphosphate kinase activity [6]. NM23 is clearly a multifunctional protein distributed in the cytosol and plasma membrane, as well as the nucleus. However, the mechanism by which NM23 suppresses tumour metastasis is still poorly understood. Detection of NM23 frequency expression in clinical specimens varied among different cancers, and no same results of prognostic analysis were achieved. It seems that the biological significance of NM23 gene expression depends on the type of neoplastic tissue [9]. The expression of the putative metastasis-suppressor gene NM23 in gastric carcinoma is controversial [2, 10]. The objective of this study was to analyze NM23 expression and its association with clinical-pathological variables of tumour, and to analyze prognostic criteria for gastric adenocarcinoma determining loco-regional node metastatic potential.

MATERIALS AND METHODS

Patients

The biopsy specimens from 56 patients (40 men, and 16 women) with invasive gastric adenocarcinoma diagnosed at the Department of Pathology, Faculty of Medicine University of Sarajevo, Bosnia and Herzegovina, from January 1999 to December 2007, were selected for this study. All clinical-pathological data are summarized according to the treatment arm in Table 1. Gastric adenocarcinoma specimens were reviewed using morphologic and immunohistochemical criteria according to the WHO classification of gastric carcinoma [11] and staged according to TNM classification [12]. All of the samples were routinely fixed in 4% buffered formalin, embedded in paraffin, and cut into 5 μm section. Four blocks from each tumour specimens were submitted for paraffin embedding, each containing as mush as possible tumour tissue with the deep advancing edge and piece of adjacent mucosa. One block of them contains pieces of morphologically normal distant mucosa, at 10 cm from the tumour.

Immunohistochemical staining

Immunohistochemical analyses of the expressions of NM23 were performed according to the routine processes. Briefly, 5-μm sections of tumour or normal tissues were mounted on poly-D-lysine coated slides. Thin sections were deparaffinized in xylene and rehydrated in a series of ethanol solutions (100%, 90%, and 80%) for 5 minutes each, washed in distilled water and 0.01 PBS (pH 7.4), immersed in 10 mmol/L citrate buffer (pH 6.0) and put in a microwave for 5 min at 60°C for antigen retrieval. Then they were placed in methanol containing 3% H_2O_2 for 30 min at 4°C to block endogenous peroxidase activity and incubated with rabbit serum for 10 min to block non-specific antibody binding sites. The primary antibody was applied at a working concentration and incubated for 2 hours at 4°C. The monoclonal antibody used is anti-human NM23 (1:50; DAKO, Denmark). This antibody has affinity for both H1 and H2 components of the NM23 protein. The secondary antibody and the avidin-biotin complex (ABC) were applied to slides. Diaminobenzidine (DAB) was used as a chromogen and sections were counterstained with Mayer's hematoxylin. Negative controls were obtained by replacing the primary antibody by non-immunized rabbit or mouse serum.

Quantification of immunostaining

Positive results were visible as yellow to brown cytoplasmic staining for the investigated antibody. The expression of the antigen was evaluated in a semiquantitative manner [13]. The criteria used to assess NM23 expression were based on the number of stained cells, and scores were assigned as follows: a) score 0<10%, score 1=10-25%, score 2= 26-50%, score 3=51% or more stained cells. Scores 0 to 2 was considered as negative (protein down-expression) and score 3 was considered as positive (protein over-expression). The sections were examined assessing the percentage of cells with positive reaction in 10 microscopic fields at 400 magnification (X400). Non-

neoplastic gastric mucosa was used as the internal positive control. Normal tonsil tissue was used as negative control.

Statistical analysis

The collected data was analysed using IBM statistics SPSS software version 19.0. Quantitative variables were expressed as means. The relationships between expression of NM23 and clinicopathological parameters (gender, age, size, tumour differentiation, lymphovascular invasion, number of lymph nodes involved, Lauren- Goseki-, or Ming classification) were analyzed by the Chi-Square test or students (t) test, respectively. Statistical analysis was performed using Kolmogorov-Smirnov and Spearman test. Probability (p-value) ≤ 0.05 was considered significant.

RESULTS

Characteristics of 56 patients with gastric cancer are shown in Table 1. Among 56 patients, mean age was 63.3 years (range 48 to 81 years); 3 (5.3 %) had tumour size pT1, 15 (26.7 %) pT2, 26 (46.4 %) pT3, and 12 (21.4%) pT4. Thirteen tumours (23.2 %) were classed as grade I and grade IV, fifteen tumours (26.7 %) at grade II and at grade III. Among 56 patients 13 (23.2 %) were pNo, 26 (46.4 %) were pN1, 9 (16.0%) were pN2, and 8 (14.2%) were pN3. Among these patients 44 (78.5%) had lymphovascular invasion, 34 (60%) had inatestinal and 22 (39.2%) had diffuse type carcinoma (Lauren classification). Thirty-five cases were Goseki 1 (62.5%), five cases was Goseki 2 (8.9%), six cases were Goseki 3 (10.7%), and ten cases were Goseki 4 (17.8%). The immunohistochemical expression of NM23 was strictly cytoplasmic. There was no nuclear staining. The intensity of staining was not considered while evaluating the expression. Positive and negative NM23 staining was identified in non-neoplastic gastric mucosa. Normal gastric epithelial cells were homogeneously stained (> 51%) by monoclonal anti NM23 antibody. This was considered as an intrinsic control although we studied positive and negative control slides. NM23 expressions in the adjancent non-neoplastic gastric mucosas were highly variable. Positive NM23 stain (score 3) (Figure 1.) was observed in 30 (53.3%) and negative stained in 26 (46.4%) cases of adjacent gastric mucosa (Figure 2.). No expression of NM23 protein in 56 (100%) gastric adenocarcinoma (Figure 3-5.). The normal gastric mucosa had the higher expression of NM23, than gastric adenocarcinoma (p=0.0001). NM23 expression was evaluated with respect to patients clinicopathological data (Table 2). NM23 showed no significant differences regarding gender (p=0.115), tumour size (p=0.844), tumour grade (p=0.172), lymphovascular invasion (p=0.606), lymph node metastases (p=0.311), Lauren- (p=0.426), Goseki- (p=0.458) and Ming classification (p=0.212). No significant correlation was found between

TABLE 1. Clinicopathological and NM23 protein immunohistochemical (*p value*) data of 56 patients with castric carcinoma.

Characteristic		Number of patients	(%)	
Age (median, range)		63 (48 - 81)		
Tumor size (pT)				
1		3	(5.3)	
2		15	(26.7)	
3		26	(46.4)	
4		12	(21.4)	
Grade:*				
1		13	(23.2)	
2		15	(26.7)	
3		15	(26.7)	
4		13	(23.2)	
Vascular invasion:				
Yes		44	(78.5)	
No		12	(21.4)	
Lymph node (pN):				
pN0		13	(23.2)	
pN1		26	(46.4)	
pN2		9	(16.0)	
pN3		8	(14.2)	
Lauren classification:				
Intestinal		34	(60.7)	
Diffuse		22	(39.2)	
Goseki classification:				
1		35	(62.5)	
2		5	(8.9)	
3		6	(10.7)	
4		10	(17.8)	
Ming classification:				
Nodular		14	(25.0)	
Diffuse		7	(12.5)	
Nodular / diffuse		35	(62.5)	
NM23†:				*p value***
Non- neoplastic mucosa	Negative	26	(46.4)	
	Positive	30	(53.5)	0.0001
Tumour	Negative	56	(100.0)	
	Positive	0	(0)	0.0001

*According to the WHO classification of gastric cancer [11].
†Staining intensity: 0<10%, 1=10-25%, 2= 26-50%, 3=>51% of stained cells. Scores 0 to 2 was considered as negative, and score 3 was considered as positive.
** Kolmogorow-Smirnov one sample test for testing uniform distribution.

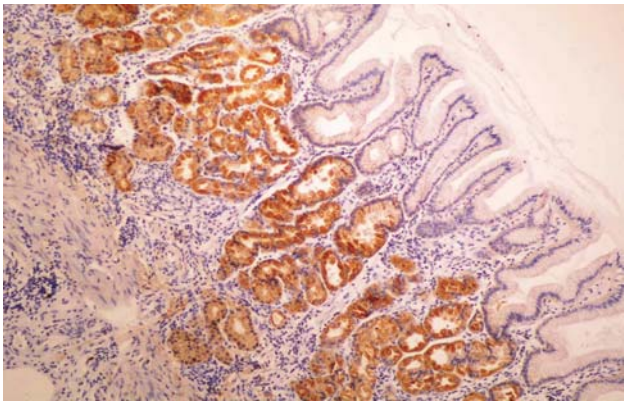

FIGURE 1. Positive cytoplasmic NM23 expression of epithelial cells in normal gastric mucosa (IH, X 250).

TABLE 2. Clinicopathological variables and their correlation with immunohistochemical expression of NM23 protein of 56 patients with castric carcinoma.

Variables	Number of patients (%)	NM23 – Number of patients Non-neoplastic mucosa positive / negative (score 0-2) /(score 3)		Neoplastic mucosa positive / negative (score 0-2) / (score 3)		*p value***
Sex:						
Male	40 (71.4)	22	18	0	40	Ro=0.213
Female	16 (28.5)	9	7	0	16	*p*=0.115
Tumor size (pT):						
1	3 (5.3)	3	0	0	3	
2	15 (26.7)	8	7	0	15	Ro=0.027
3	26 (46.4)	13	13	0	26	*p*=0.844
4	12 (21.4)	8	4	0	12	
Grade:*						
1	13 (23.2)	9	4	0	13	
2	15 (26.7)	8	7	0	15	Ro=-0.185
3	15 (26.7)	9	6	0	15	*p*=0.172
4	13 (23.2)	4	9	0	13	
Vascular invasion:						
Yes	44 (78.5%)	24	20	0	44	Ro=-0.070
No	12 (21.4%)	6	6	0	12	*p*=0.606
Lymph node (pN):						
pN0	13 (23.2)	8	5	0	13	
pN1	26 (46.4)	14	12	0	26	Ro=0.138
pN2	9 (16.0)	5	4	0	9	*p*=0.311
pN3	8 (14.2)	4	4	0	8	
Lauren classification:						
Intestinal	34 (60.7)	20	15	0	34	Ro=0.108
Diffuse	22 (39.2)	9	13	0	22	*p*=0.426
Goseki classification:						
1	35 (62.5)	20	15	0	35	
2	5 (8.9)	2	3	0	5	Ro=-0.101
3	6 (10.7)	1	5	0	6	*p*=0.458
4	10 (17.8)	4	6	0	10	
Ming classification:						
Nodular	14 (25.0)	6	8	0	14	Ro=0.121
Diffuse	7 (12.5)	5	2	0	7	*p*=0.212
Nodula/diffuse	35 (62.5)	18	17	0	35	

*According to the WHO classification of gastric cancer [11] .
** Spearman rank correlation test according to NM23.

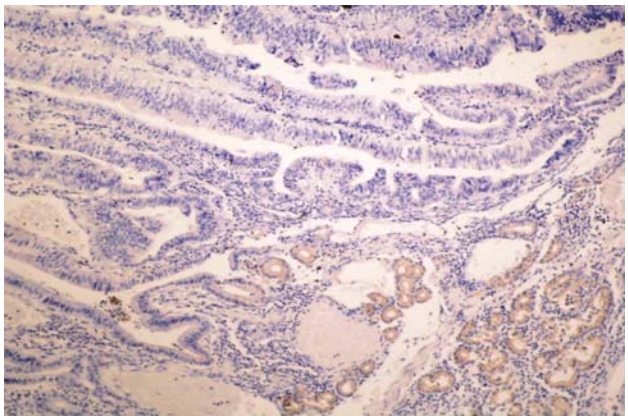

FIGURE 2. Absent NM23 expression in gastric adenocarcinoma cells (left and up) and positive in adjancent non-neoplastic gastric mucosa (IH, X 250).

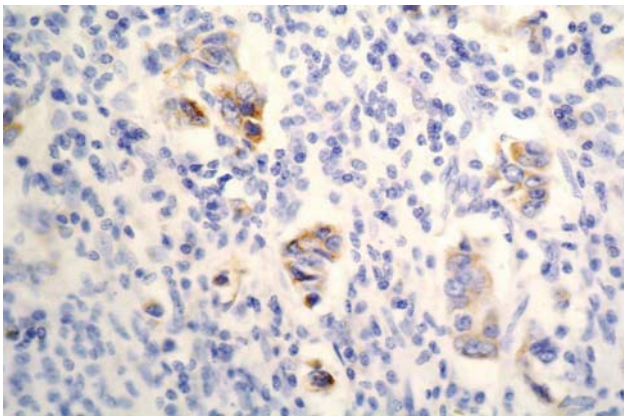

FIGURE 3. Negative NM23 expression (score 2) in gastric adenocarcinoma cells (IH, X 400).

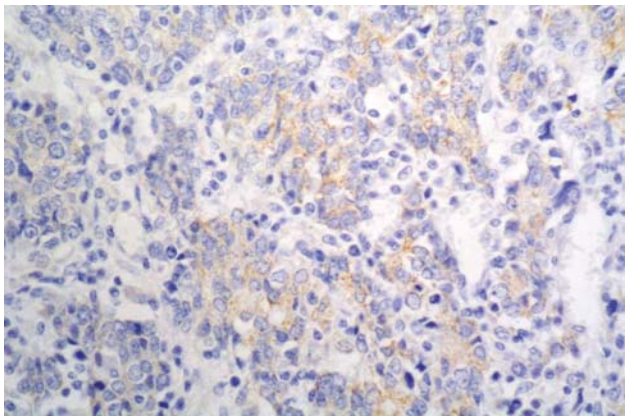

FIGURE 4. Negative NM23 expression (score 1) in gastric adenocarcinoma cells (IH, X 400)

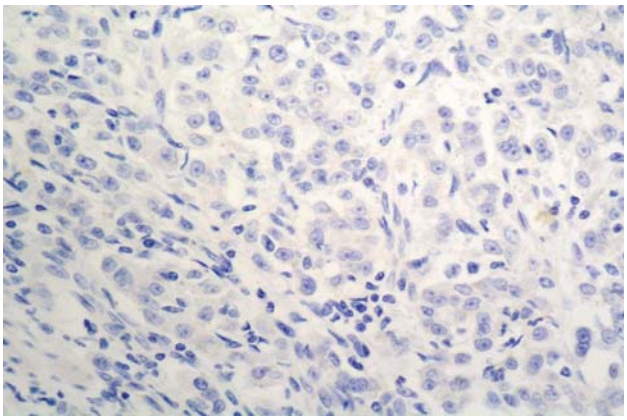

FIGURE 5. Negative NM23 expression (score 0) in gastric adenocarcinoma cells (IH, X400).

NM23 expression and analysed clinicopathologic factors. Table 2. summarized the data with statistical analyses.

DISCUSSION

Two highly homogenous genes, NM23-H1 and NM23-H2, have been described, both located on the long arm of chromosome 17, coding for the 18.5 an 17 kD proteins respectively. NM23 protein is a metastasis suppressor protein, expressed in all cellular compartments. *In vitro* correlates of suppression include reduced invasion, motility and soft agar colonization, and induction of differentiation. NM23 expression has been widely studied in various cancers and with their relation to staging and prognosis. NM23 expressions are generally, but not uniformly associated with improved prognosis in various type of carcinomas. Expression of NM23 has been shown to be inversely correlated with the metastatic potential of several human cancers. Reduced expression of NM23 in breast, hepatoecellular and ovarian carcinoma correlates with increased metastatic potential [14-18], but in oesophageal squamous cell, prostate and lung carcinoma, disease progression is associated with increased NM 23 gene expression [8,19,20]. The relatively large number of studies analysed NM23 protein in colorectal carcinoma [13], but a small number of them analysed this protein in gastric carcinoma [2, 14, 21]. In the present study expression of NM23 protein was observed in normal gastric mucosa in 53.5% of cases with strong diffuse cytoplasmic staining. We observed a similar percentage (46.4%) of cases with negative staining in adjacent non-neoplastic mucosa. There were some differences about expression of NM23 in non-neoplastic mucosae in adjacent gastric cancer between different persons. When compared the specimens between the two groups, NM23 expression did not demonstate significant correlation. Our results do not support findings of Muta's study. Muta analyzed gene and protein expression of NM23, using Northern blot and immunohistochemical techniques [22]. He noted that expression of NM23 protein in tumour tissue was higher than those in the corresponding normal mucosae. This suggests a linkage of NM23 in the process of the gastric cancer progression. Our results suggest that biological significance of NM23 expression may be quite different in the same organ. Neoplastic gastric tissue showed negative expression of NM23, suggests that absent staining in gastric adenocarcinoma was associated with disease progression, but these mechanism is not understood and remain to be determined conclusively. In our series, the analysis of NM23 expression revealed a higher tumour grade, higher incidence of metastatic lymph nodes, higher intestinal type of tumours according to Lauren classification, higher Goseki type 1 tumours and higher nodular/diffuse type of tumours (Ming classification), and advanced pT categories in patients without protein expression, although this result did not reach statistical significance. This result suggested that loos of NM23 expression in gastric carcinoma tissue may had relation with development, progression, invasion and metastasis of neoplasm. This finding suggests a potential protective effect of this protein in tumour genesis. This study indicates a complex role of NM 23 in gastric cancer and may not solely function as tumour suppressor protein as commonly perceived. Our results do not support findings of similar studies. There were also some discrepancies amnog previous studies of the same tumours [2, 10, 21, 23]. Lee et al. analysed the relationship of p53, nm23, PCNA and HER-2 with clinicopathological parameters in gastric cancer and the survival results [2]. He concluded that expression of NM23 and p53 was related with poor prognosis of gastric cancer. Monig et al. analysed clinical significance of NM23 gene expression in gastric cancer [10]. Their series did not show a correlation of protein expression in neoplastic gastric tissue in terms of lymph node and distant metastasis or prognosis in gastric cancer patients. Yeung [21] suggested that NM23 may have a role

in gastric carcinoma pathogenesis, but do not show a correlation with metastasis. Muller [23] analysed NM23 expression and prognostic impact in 413 gastric carcinoma. Expression of NM23 was detected in 84.5% (n=349) of all tumours and demonstrated positive correlation with the intestinal type of tumour, according to the Lauren classification and advanced pT categories, and was also correlated with the presence lymphatic vessel invasion. No correlation is demonstrated between NM23 expression and lymph node involvement. Results of this study showed that expression of the NM23 metastasis suppressor gene is correlated with aggressive tumour growth and poor prognosis but it is not an independent prognostic marker. Nakamura [24] has found expression of NM23 in 24 out of 31 cases of gastric carcinoma. They results suggest that expression of this protein is correlated to tumour progression and / or proliferation rather than the suppression of metastasis. Such a variation may be due to heterogeneity of primary tumour distribution, methods of investigation and scoring systems for pathological variables.

CONCLUSION

In conclusion, our data of NM23 expression did not show a significant correlation between analysed clinical-pathological variables, but these results suggest that protein NM23 may have a role in gastric carcinoma pathogenesis. It is possible that NM23 could play variable roles in different molecular events and contribute to distinct outcome. The detailed biological roles of this marker in gastric carcinogenesis require further investigation.

DECLARATION OF INTEREST

The authors declare no conflict of interest for present study.

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