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ORIGINAL ARTICLE

Immunohistochemical localization of the NM23 protein in salivary gland neoplasms with distinct biological behavior

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Abstract The NM23 protein was shown to be associated with metastasis suppression in human malignancies with various tissue origins. However, its association with the metastatic phenotype of salivary gland neoplasms (SGN) remains unknown. To evaluate the role of NM23 in SGN, the expression patterns of NM23 in the following were compared: benign (pleomorphic adenoma) vs malignant (adenoid cystic carcinoma and mucoepidermoid carcinoma) SGN, and primary malignancies with/without evidence of metastasis vs their metastatic implants (MI). The lesions were studied immunohistochemically. NM23 protein was found in the cytoplasm of 75% of benign SGN, 73.3% of primary SGN malignancies with no evidence of metastasis, 86.6% of primary SGN malignancies with evidence of metastasis, and 60% of MI. There was no statistically significant difference in the frequency of NM23-positive cells between benign and primary malignant tumors (p= 0.79), nor between primary malignancies with/without evidence of metastasis and MI (p=0.51). However, nuclear NM23 protein was restricted to primary SGN malignancies with evidence of metastasis and MI. The presence of nuclear NM23 protein may be a good marker for predicting the metastatic potential of SGN malignancies.

Keywords NM23 · Salivary gland tumors · Metastasis · Prognosis · Biological behavior

The experimental analyses of this work were performed at the Oral Pathology Laboratory of the Federal University of Uberlândia, Brazil.

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Introduction

Metastasis is the most dreadful biological hallmark of malignant tumors and the primary cause of death in cancer patients. Salivary gland malignancies are slow-growing tumors, which eventually will develop metastasis if left untreated. However, the timing of metastasis of these malignancies varies from patient to patient. It is important to determine the metastatic potential of these tumors at the time of diagnosis because of the nature of these slow-growing tumors. Histologic grading was considered a useful parameter to predict the metastatic potential of malignancies [4, 7, 10]. However, current parameters may

not be completely reliable. It is necessary to search for new biomarker(s) capable of providing a better prediction of the metastatic potential of malignant SGN.

Constitutive expression of the *NM23*gene was shown to suppress metastasis in vitro as well as in human tumor cohorts [9, 14, 25, 29]. Moreover, decreased levels of NM23 protein was shown to be associated with aggressive biologic behaviors such as a reduced disease-free period, a poor overall survival rate, and lymph node metastasis. NM23 was also correlated with poorly differentiated grades of melanoma and malignancies of the breast, stomach, ovary, cervix, and liver [8, 14–16]. However, this inverse correlation is not consistently seen in malignancies of all tissue origins. In neuroblastoma and pancreatic carcinoma, an opposite trend was reported, while in other malignancies, such as colorectal, thyroid, and lung carcinomas, it does not correlate with neoplastic aggressiveness [3, 12, 14].

An inverse correlation between NM23 expression and the metastatic potential of adenoid cystic carcinoma and mucoepidermoid carcinoma of the salivary glands was previously reported [17, 28, 31]. In addition, positive NM23 immunostaining is more frequently seen in benign SGN, when compared to malignant SGN [13]. However, a recent study showed no correlation between NM23 immunostaining and the biological behavior of salivary gland adenocarcinomas [5]. These different results have led to some controversy regarding the role of NM23 in SGN and while these differences may be due to either the antibody used, the immunohistochemical methods or the tissue specificity, there remains a question regarding the linkage of NM23 to SGN metastatic potential. In this study, we analyzed SGN specimens to better understand the role of NM23 in the metastatic potential of SGN.

To evaluate whether NM23 could be a biomarker for predicting the metastatic potential of SGN, immunolocalization of NM23 was characterized in SGN exhibiting different biological behaviors: (a) to quantify and compare the NM23 immunostaining indices of various SGN whose biologic behavior ranges from benign to distant metastasis, and (b) to evaluate whether the NM23 immunostaining index could be used to discriminate primary malignancies with no evidence of metastasis from primary malignancies with evidence of metastasis.

Materials and methods

The present study was approved by the Committees for Ethics on Research of the institutions where it was performed.

Fifty-six samples of salivary gland tumors were collected from the archives of the Oral Pathology Service of the Federal University of Uberlândia and the Center of Research and Training for Cancer A. C. Camargo (all institutions from Brazil). There were 16 pleomorphic adenomas (PA), 21 adenoid cystic carcinomas (ACC) and 19 mucoepidermoid carcinomas (MEC). All of the cases were reviewed and categorized according to the WHO classification of salivary gland tumors [2].

Medical records of the patients were reviewed and clinic-pathological data were collected. Evidence of metastasis was confirmed with one or more of the following studies: radiographs, computerized tomography scan or magnetic resonance image; and/or histological evidence of local/distant metastases. The mean follow-up period was 6.9±5.7 years with a range from 2 to 31 years. For analytic purposes, four groups were delineated according to the observed biological behavior of the lesions. These four groups are benign (n=16), primary malignancies with no evidence of metastasis (n=6 ACC and 9 MEC), primary malignancies with evidence of metastasis (n=10 ACC and 5 MEC), and metastatic implants (n=5 ACC and 5 MEC). There were five pairs of ACC and four pairs of MEC sampled from the primary sites and their metastatic implants. Most of the malignant lesions were located in major salivary glands and had clinically advanced staging as shown in Tables 1, 2 and 3.

Avidin–biotin–peroxidase immunohistochemical technique was employed to localize the distribution of NM23 in the tissue specimens. Briefly, 3 μm formalin-fixed paraffin tissue sections were mounted on 3-aminopropyltriethoxysilane (Sigma-Aldrich, USA) coated slides and the antigens of interest were retrieved with EDTA (1 mM, pH 8.0) in a microwave environment. Endogenous biotin and peroxidase activity was blocked with biotin blocking system (Dako Cytomation, USA) and H_2O_2 solution, respectively. The primary monoclonal antibodies used to detect the NM23 antigen were NCLnm23-37.6 and NCLnm23-2-NM301 (Novocastra Labs., Newcastle, UK) at 1:600 and 1:400 dilutions, respectively. After overnight incubation in a humidified chamber at room temperature, the antibody binding sites were visualized with the chromogen diaminobenzidine (Sigma-Aldrich, USA) and the nuclei were counterstained with Meyers' hematoxylin. Negative control was performed as above, without primary antibody. As a positive control, tissue fragments of an infiltrating ductal carcinoma of the breast known to be strongly NM23-positive were used. Fragments of non-neoplastic salivary glands were also used to evaluate the distribution of NM23 in normal salivary gland tissue.

The presence and distribution of chromogen precipitation in the tissue, as well as the pattern of its distribution were recorded. For purpose of quantitation, an immunostaining index was scored in each sample where the index is the percentage of cytoplasmic NM23-positive cells in an area of 1,000 neoplastic cells [19]. Correlation between the immunostaining indices using the two primary antibodies in this study was determined by the Spearman test. The mean

Table 1 Clinical features, histopathology and NM23 immunostaining index of the primary malignancies with no evidence of metastasis investigated in the present study

Case	Diagnosis	Primary site	TNM staging	Histological pattern	Index of NM23 immunostaining (%)
1	ACC	Parotid gland	IV	Cribriform	31.2
2	ACC	Parotid gland	II	Cribriform/tubular	15.5
3	ACC	Submandibular gland	IV	Cribriform	0.0
4	ACC	Submandibular gland	IV	Cribriform/tubular	0.0
5	ACC	Maxilla	III	Cribriform	0.0
6	ACC	Palate	III	Cribriform/tubular	35.5
7	MEC	Parotid gland	N/A	Low grade	62.4
8	MEC	Parotid gland	IV	High grade	60.2
9	MEC	Parotid gland	II	Low grade	86.9
10	MEC	Parotid gland	IV	Low grade	8.3
11	MEC	Submandibular gland	IV	Low grade	0
12	MEC	Palate	IV	Moderate grade	3.4
13	MEC	Maxilla	IV	Low grade	65.8
14	MEC	Posterior mandibular ridge	N/A	High grade	88.9
15	MEC	Posterior mandibular ridge	IV	Low grade	14.7

ACC=adenoid cystic carcinoma, MEC=mucoepidermoid carcinoma, N/A=not available

Table 2 Clinical features, histopathology and NM23 immunostaining index of the primary malignancies with evidence of metastasis investigated in the present study

Case	Diagnosis	Primary site	TNM staging	Histological pattern	Site of metastasis and/or corresponding case in Table 3	Index of NM23 immunostaining (%)
16	ACC	Submandibular gland	IV	Cribriform	Lung, refer to case #31	0.0
17	ACC	Parotid gland	IV	Tubular	Lymph node, refer to case #32	54.2
18	ACC	Submandibular gland	IV	Cribriform	Lung, refer to case #33	16.3
19	ACC	Submandibular gland	IV	Cribriform	Lung, refer to case #34	1.3
20	ACC	Submandibular gland	IV	Cribriform	Lung, refer to case #35	6.1
21	ACC	Submandibular gland	IV	Solid	Lymph node	2.3
22	ACC	Submandibular gland	II	Cribriform	Lymph node	0.0
23	ACC	Submandibular gland	IV	Solid	Lymph node	43.9
24	ACC	Submandibular gland	IV	Solid	Lymph node, lung	95.5
25	ACC	Submandibular gland	IV	Cribriform	Lung	8.0
26	MEC	Parotid gland	IV	High grade	Lymph node, refer to case #36	72.9
27	MEC	Parotid gland	IV	High grade	Lymph node, refer to case #37	51.8
28	MEC	Floor of the mouth	IV	High grade	Lymph node, refer to case #38	83.6
29	MEC	Parotid gland	IV	High grade	Lymph node, refer to case #39	5.1
30	MEC	Parotid gland	IV	High grade	Lymph node	93.4

ACC=adenoid cystic carcinoma, MEC=mucoepidermoid carcinoma

Table 3 Clinical features, histopathology and NM23 immunostaining index of the metastatic implants investigated in the present study

Case	Diagnosis	Location	Histological pattern	Primary site and/or corresponding case in Table 2	Index of NM23 immunostaining (%)
31	ACC	Lung	Solid	SMG, refer to case #16	13.8
32	ACC	Lymph node	Solid	SMG, refer to case #17	0.0
33	ACC	Lung	Cribriform	SMG, refer to case #18	27.2
34	ACC	Lung	Cribriform	SMG, refer to case #19	15.7
35	ACC	Lung	Solid	SMG, refer to case #20	0.0
36	MEC	Lymph node	High grade	PG, refer to case #26	0.0
37	MEC	Lymph node	High grade	PG, refer to case #27	55.6
38	MEC	Lymph node	High grade	FOM, refer to case #28	16.6
39	MEC	Lymph node	High grade	PG, refer to case #29	60.0
40	MEC	Lymph nodes	High grade	PG	10.2

ACC=adenoid cystic carcinoma, MEC=mucoepidermoid carcinoma, SMG=submandibular gland, PG=parotid gland, FOM=floor of mouth

NM23 immunostaining indices were compared between the four delineated groups by Mann–Whitney U or Kruskal–Wallis non-parametric statistical tests. Only probabilities of alpha-error less than 0.05 were considered significant.

Results

Immunostaining intensity with the antibody NCLnm23-37.6 was stronger than that with NCLnm23-2-NM301 regardless of the histologic typing of the SGN. There was a strong correlation between the indices of immunostaining reaction obtained with these two antibodies ($p<0.001$). The data presented in this report were the results of NCLnm23-37.6.

The NM23 protein was primarily seen in the cytoplasm of the salivary gland cells. In PA, both luminal and non-luminal (myoepithelial) cells were positive for NM23, but a stronger immunostaining was observed in the luminal cells (Fig. 1). In MEC, a strong NM23 immunostaining was seen in the intermediate and luminal cells of the cystic and solid areas. The mucous, clear and caliciform cells were negative (Fig. 2). In ACC, NM23 was observed in both luminal and non-luminal cells, albeit a strong reaction was restricted to the luminal cells in a fashion similar to that seen in PA (Fig. 3). The staining was also more intense in the cells of solid areas (not shown).

Due to the rarity of SGN malignancies, only ACC and MEC were available for evaluation at the time of this study. For convenience, "malignant SGN" and "ACC and MEC" will be used interchangeably in this report despite the fact that there are malignant SGN other than ACC and MEC.

The clinical, histopathologic and immunostaining data of the investigated cases are presented in Tables 1, 2 and 3. In summary, cytoplasmic NM23 was present in 12 out of 16 (75.0%) of the PA (mean index=29.4±30.3%) and in 24 out of 30 (80.0%) of the primary malignant SGN (mean

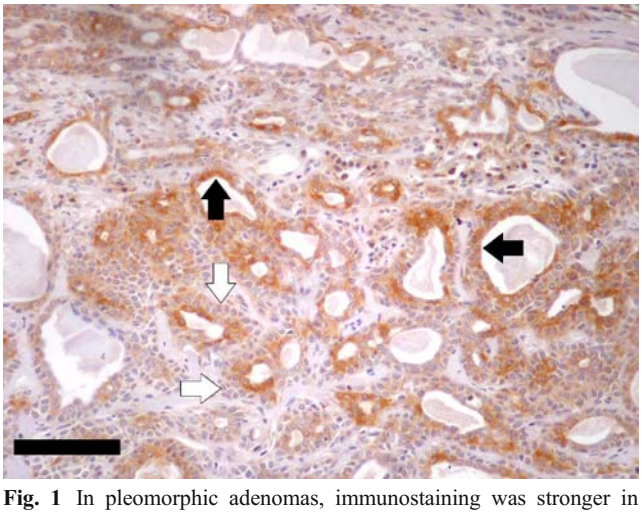

Fig. 1 In pleomorphic adenomas, immunostaining was stronger in luminal cells (black arrows) than in myoepithelial, non-luminal (white arrows) cells. Streptavidin–biotin–peroxidase, scale bar=100 μm

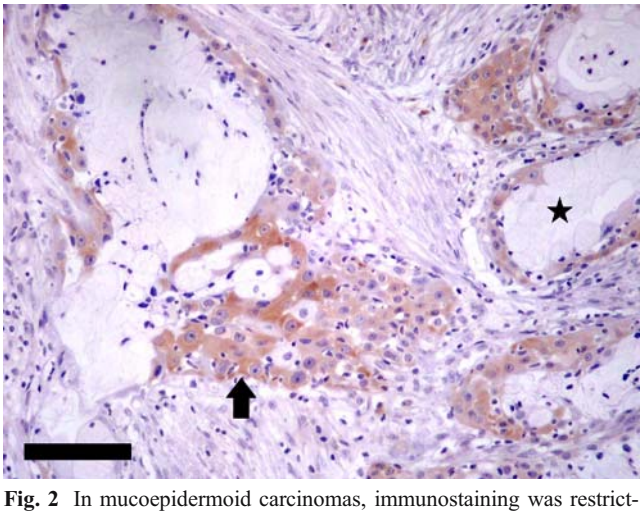

Fig. 2 In mucoepidermoid carcinomas, immunostaining was restricted to epidermoid and intermediate cells (arrows) while mucous and clear cells were negative (star). Streptavidin–biotin–peroxidase, scale bar=50 μm

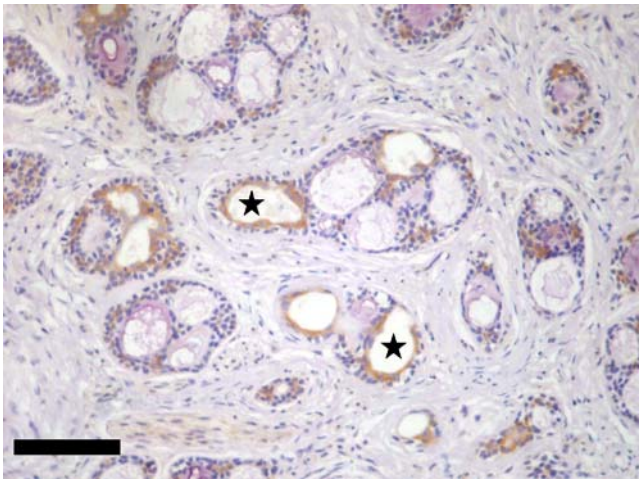

Fig. 3 In this cribriform adenoid cystic carcinoma, NM23 immunostaining was more evident in the cells underlying the luminal spaces (stars). Streptavidin–biotin–peroxidase, scale bar=100 μm

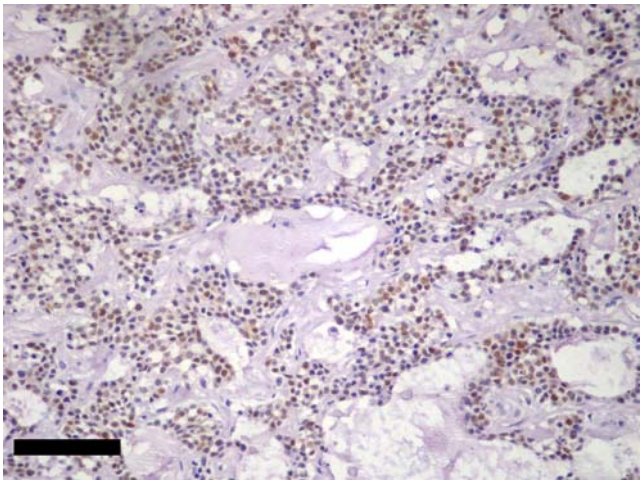

Fig. 4 Nuclear NM23 immunolocalization was observed in this adenoid cystic carcinoma. Streptavidin–biotin–peroxidase, scale bar=100 μm

index=33.6±34.4%). Among these 24 out of 30 malignancies, NM23 was found in 11 out of 15 (73.3%) of the primary malignancies with no evidence of metastasis (mean index=31.5±32.9%), and in 13 out of 15 (86.7%) of the primary malignancies with evidence of metastasis (mean index=35.6±36.9%). In addition, NM23 was found in six out of ten (60.0%) metastatic implants (mean index=18.9±22.5%). Statistically, there was no significant difference of the NM23 immunostaining index between benign and primary malignant tumors (p=0.79), nor among primary malignancies with no evidence of metastasis, primary malignancies with evidence of metastasis, and their metastatic implants (p=0.51). However, there is a trend showing a decreased NM23 expression in metastatic implants compared to their primary lesions. This trend can be seen in ACC (mean index=11.3% vs 22.8%) and MEC (mean index=26.4% vs 61.4%).

It is interesting to note that NM23 protein was seen in the nuclei of the neoplastic cells in 10 out of 15 primary malignancies with evidence of metastasis (four out of five MEC and six out of ten ACC) and their metastatic implants (six out of ten cases) (Fig. 4). This pattern was not observed in PA and primary SGN malignancies without evidence of metastasis.

Discussion

The NM23 protein (or nucleoside-diphosphate kinase) was associated with metastasis suppression experimentally [29]. Clinic-pathologically, there were reports that decreased or negative immunostaining of this protein may be associated with the development of metastasis of human malignancies from various tissue origins [8, 14, 25, 30]. However, this inverse relationship in salivary gland neoplasms has been controversial. It was found conclusively in some studies [11, 17, 28, 31] but inconclusively in one study [5]. In the present study by using an alternative, behavior-driven method to revisit this issue, we did not find any correlation between the metastatic potential of SGN and NM23 protein. Nevertheless, we did observe two interesting trends: decreased cytoplasmic NM23-positive cell populations in metastatic implants compared to their primary counterparts and nuclear NM23-positive cells solely seen in lesions with a history of metastasis and their metastatic implants.

In the present study, NM23 protein was immunohistochemically detected in 75.0% of PA, 68.7% of ACC, and 92.8% of MEC. These findings are similar to those reported previously [17, 28]. Of those 56 SGNs studied, the NM23 protein was found to be located in the cytoplasm of 75% of benign tumors, 73.3% of primary malignancies with no evidence of metastasis, 86.6% of primary malignancies with evidence of metastasis, and 60% of metastatic implants. Statistical analysis did not demonstrate a consistent correlation between NM23 expression and the metastatic potential of SGN. Our findings were consistent with a previous report [5]. However, it was reported that negative NM23 immunostaining in malignant SGN is associated with a higher frequency of metastasis [17, 28]. A recent study showed that benign SGN were significantly associated with more frequent NM23 immunostaining reactions compared to the malignancies of SGN [13]. These contradictions illustrate that the interpretation of NM23 as a biological behavior marker in SGN remains elusive.

One possible explanation for the discrepancies is the diversity of the NM23 family combined with the selection of particular detecting antibodies. The NM23 family consists of 8 members, namely, NM23-H1 to NM23-H8.

NM23-H1 and NM23-H2 are abundant and most studied [1, 6, 20, 26]. Historically, several antibodies raised against NM23 were analyzed with respect to a wide range of neoplasms [5, 12, 16, 22, 23, 27]. Many studies were completed before the characterization of all members of the NM23 family and consequently it is not clear which family member was identified. Thus, it is difficult to interpret and compare the results. To make sure that the distribution of NM23 in this study was representative, we employed two clones of antibody available commercially: NCLnm23-37.6 and NCLnm23-2-NM301. The 37.6 monoclonal antibody binds to the H1 isoform of NM23, but may cross-react with the H2 isoform, while the NM301 clone is specific for binding to the H1 isoform only (information obtained from Novocastra Labs., Newcastle, UK). In the present work, less intense immunostaining was obtained with the NM301 clone, but similar histo-morphological distribution of NM23-positive cells was observed using both antibodies, resulting in a strong statistical correlation between the immunostaining indices of these two antibodies. Similar result was also found in colorectal carcinomas [18]. Thus, we believed that the distribution of NM23 protein in the SGN of this study is representative and the data of this study are reliable.

Many qualitative and semiquantitative methods for the evaluation of NM23 immunostaining were applied to the study of salivary gland neoplasms. In common, all of these protocols established empiric and heterogeneous cut-off values to determine positivity or negativity [5, 12, 17, 27, 28, 31]. Yet, in our study there was no empiric cut-off value. Only those with immunostaining indices of zero were considered negative. Our results are not consistent with the conclusions of four different groups [13, 17, 28, 31]. This discrepancy may be due to different data analysis methods. Therefore, our data were re-analyzed with two different alternatives: by tumor growth pattern and by overexpressed cell population. Clusters of lesions with distinct histomorphology growth pattern (e.g. cribriform vs solid patterns) believed to be associated with certain biological behavior were compared with the NM23 expression levels of the clusters. This method was unable to demonstrate any conclusive differences among the studied lesions. Furthermore, it is debatable for almost all immunohistochemical evaluations if the analysis should be best based on the number of cells with over-expression regardless of the overall staining index, or based on the overall staining index regardless of the local areas of over-expression [15, 16, 21, 24]. With this in mind, each case was re-evaluated and re-defined using the positive reaction at three different cut-off values where more than 30%, 25%, or 10% of cells demonstrate over-expression. None of these evaluations reached different conclusions (data not shown).

Although our data did not support an inverse relationship between NM23 and the metastatic potential of SGN, there were trends showing (a) cytoplasmic NM23-positive cell populations in metastatic implants were decreased compared to their primary counterparts and (b) nuclear NM23 was exclusively seen in the primary SGN with evidence of metastasis and their metastatic implants. This nuclear NM23 staining pattern was consistent with those findings in normal and neoplastic thyroid [3], as well as cutaneous fibrohistiocytic tumors [21]. A recent report claimed that NM23-H1 and NM23-H2 are transiently internalized into the nuclei late in the G1 phase of the cell cycle, and are excluded from the nuclei during the S phase [6]. This cell cycle-dependent NM23 localization would explain the interesting trends seen in this study. It is possible that in our model, the NM23 protein in SGN malignant cells was internalized from the cytosol into the nucleus of those cells capable of metastasis. The amount of internalized NM23 is likely increased above the baseline to a level detectable by immunohistochemistry. As a result, there were decreased cytoplasmic NM23-positive cell populations and nuclear NM23 was seen solely in the metastasizing/metastatic cells. The cause-and-effect of this above baseline internalization of NM23 in metastasizing/metastatic cells is beyond the scope this study. It could be the over-internalized nuclear NM23 that triggers the metastasis cascade or metastasizing/metastatic cells to allow more NM23 molecules to be accumulated intra-nuclearly. One way or the other, it is clear that there is an above normal amount of nulcear NM23 seen only in malignant cells of primary SGN with evidence of metastasis and in cells of metastatic implants. Thus, this nuclear NM23 could be a good marker for identifying SGN neoplastic cells capable of metastasis. This interpretation is based on a small sample size. In the future, further study on a larger scale will shed more light on the potential application of nuclear NM23 protein.

In conclusion, our results demonstrate that the level of cytoplasmic NM23 protein was not associated with the metastatic potential of SGN. However, the exclusive presentation of NM23 protein in the nuclei of metastasizing/metastatic cells suggested that the nuclear NM23 protein could be a biomarker for SGN capable of metastasis.

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