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Role of nm23 expression in neuroblastoma determining prognosis and metastatic potential

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Objective: Nm23 gene family is associated with metastasis suppression and differentiation with known 4 types. Nm23 genes are expressed in different tumors where their levels are associated with reduced or increased metastatic potential. Overexpression of wild type nm23-1 proteins is defined to stimulate differentiation in neuroblastoma cell lines. The aim of this study is to search for prognostic role and metastatic importance of nm23 in neuroblastomas.

Study design: Specimens of 48 neuroblastoma cases were examined for nm23 antigen, immunohisto-chemically. Its prognostic role and relation with other prognostic factors were searched, statistically.

Results: The mean age was 34 months (1-83 months). Twenty-five cases were stage IV (52.1 %), while 23 were (47.9 %) stage I, II, and III. Fourteen (29.2 %) were in favorable histological category, and 34 (70.8 %) were unfavorable. Nm23 expression was observed in 22 (45.8 %) cases. Stage was negatively related with nm23 (p=0.006), and nm23 was an independent factor for stage (p=0.025). Nm23 was not a predictor for survival (p=0.82).

Conclusion: These results suggested that negativity of nm23 expression might be a predictor for metastasis in neuroblastomas but not an independent prognostic factor.

Keywords: Neuroblastoma, nm23, prognosis.

Introduction

Neuroblastomas account for 8- 10 % of all childhood cancers. Pathogenesis is unknown. It is one of the small blue round cell neoplasms of childhood, evolving from postganglionic sympathetic cells.[1] Histopathological classification depends on prognosis. Prognostic evaluation of neuroblastic tumors as favorable or unfavorable, according to the International Neuroblastoma Pathology Classification is called Shimada System.[2,3]

Nm23 gene family is associated with metastasis suppression and differentiation. Nm23-1 and nm23-2 are well known to be putative metastasis suppressor genes. There are 4 types defined. Nm23-H1, nm23-H2, Dr-nm23 and nm23-H4 genes encode nucleoside diphosphate (NDP) kinase.[4]

These genes are expressed in different tumor types where their levels are alternatively associated with reduced or increased metastatic progressive potential such as rectal cancer,[5] nasopharyngeal carcinoma,[6] serous ovarian carcinoma,[7,8] thyroid carcinoma,[9] lung cancer,[10] breast cancer[11] and retinoblastoma.[12]

It has been suggested that nm23 immunoreactivity might be a prognostic and differentiating factor in neuroblastoma.[13,14] Overexpression of wild type nm23-1 proteins was defined to stimulate differentiation in neuroblastoma cell lines.[15] Nm23 gene has been documented as metastasis-suppressor gene in normal development and differentiation.[16] The point mutation serme 120 to glycine in the human NDP kinase A has been

identified in several aggressive neuroblastomas, mutated proteins might be related to the aggressiveness of neuroblastoma.[17] Increased nm23-H1 copy number was thought to be a predictor for poor prognosis independently, in a series of 154 neuroblastoma cases.[18]

The prognostic role of nm23 and relationship with other prognostic factors such as age, sex, stage and differentiation, modified Shimada classification, prognosis, and survival have not been determined in neuroblastoma tissue sections. The aim of this study is to correlate these parameters with immunohistochemical expression of nm23.

Material and Methods

Neuroblastoma resection or thru-cut biopsy specimens from 48 cases diagnosed, treated and had been followed in Children's Education and Research Hospital by Oncology Study Group between 1991 and 2001. Patients had a mean 3.5 years of follow-up. The cases were staged according to the International Neuroblastoma Staging System (INSS). They were treated by surgery, chemotherapy, and/or radiotherapy according to the individual features.[3]

Formalin-fixed and paraffin-embedded, well-preserved tissue blocks of tumors were used for immunohistochemical (IHC) study. All tumor samples used for this study have been obtained at diagnosis prior to the treatment. IHC was performed by streptavidin-biotin peroxidase method. The 3µ sections were deparaffinized in xylene after keeping slides at 60°C overnight. Rehydration was done in decreasing alcohols. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 15 minutes. The sections were heated in citrate buffer (pH=6.0) in microwave for 3 times of 5 minutes. Nm23- NDP kinase Ab-1 (1/25 diluted, Neomarkers, USA) was applied as primary antibody. It is a rabbit polyclonal antibody that recognizes the products of nm23-H1 and H2. Immunohistochemical expression of anti-nm23 polyclonal antibody recognizes the product of cDNA. The epitope is a 86-102 and molecular weight of the antigen is 17kDa and 185kDa. Secondary antibodies were applied and DAB was used as a chromogen. Invasive ductal carcinoma of breast was used as a positive control and cytoplasmic staining was considered as positive. The nm23 expression was graded as negative and positive (Figure 1). We used

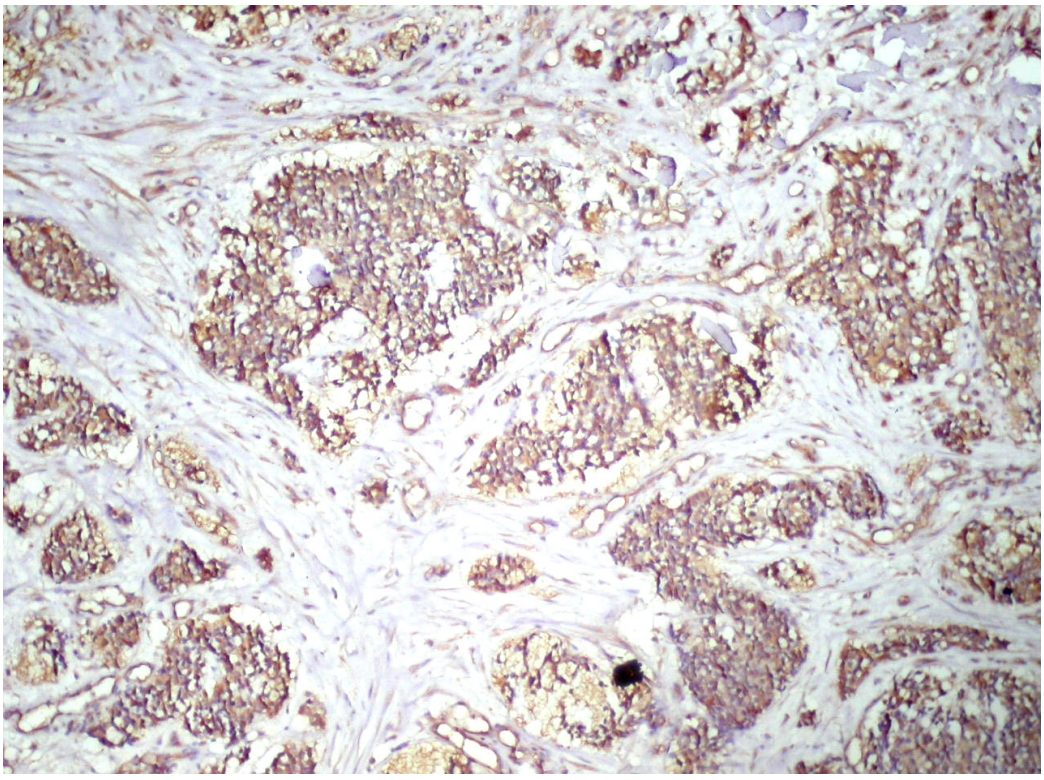

Figure 1. Diffuse nm23 expression in neuroblastoma (x40)

negative control reagent for nm23 (DAKO, N1699, USA). The evaluation was made being unaware of any of the clinical features.

Pearson correlation analysis, Kaplan-Meier method for survival curves and Log-rank test for the comparison between groups were performed for statistical analysis. P values less than 0.05 was considered to be statistically significant.

Results

The study included 48 neuroblastoma cases. The mean age was 34 months (1–83 months). Twenty-five were male (52.1%) and 23 were female (47.9%). Twenty-five were stage IV (52.1%), while 23 were (47.9%) stage I, II, and III. Only 6 of the cases were located at mediastinum, others were originated from adrenals. Twenty-two of the cases died of disease (45.8%). Twenty-six of them were alive. Thirty-two of the cases (66.7%) were considered well response to chemotherapy, other were poor in response, except one case which had died in 1 month after diagnosis before enough time to evaluate chemotherapy effect. According to the modified Shimada classification, 12 cases (25%) were differentiated, 14 (29.2%) were poorly differentiated, and 22 (45.8%) were undifferentiated. Fourteen cases (29.2%) were of favorable histology category, and 34 (70.8%) were unfavorable. Necrosis was observed in 19 cases (39.6%) and calcification in 5 cases (10.4%). Nm23 expression was observed in 22 (45.8%) cases, and 26 (54.2%) were negative. All of the differentiated cases showed positivity for nm23 in neurophilic substance and in the cytoplasm of a few ganglion-like cells.

Mean survival time was 27.54 months. Mean follow-up time was 43 months. There was no specific sex distribution between nm23 positive and negative cases. Age, existence of necrosis and calcification of the tumor, mean survival time status of favorable or unfavorable histology did not correlate with nm23 expression. Stage was negatively related with nm23 immunoreactivity (p=0.006) (Figure 2), and nm23 was an independent predictor for stage (p=0.025). Nm23 expression was not a predictor for survival (p=0.82) (Figure 3). Properties of the cases according to nm23 status is given in Table 1.

Discussion

There are many prognostic factors considered for neuroblastoma. There is an evolving list of prognostic

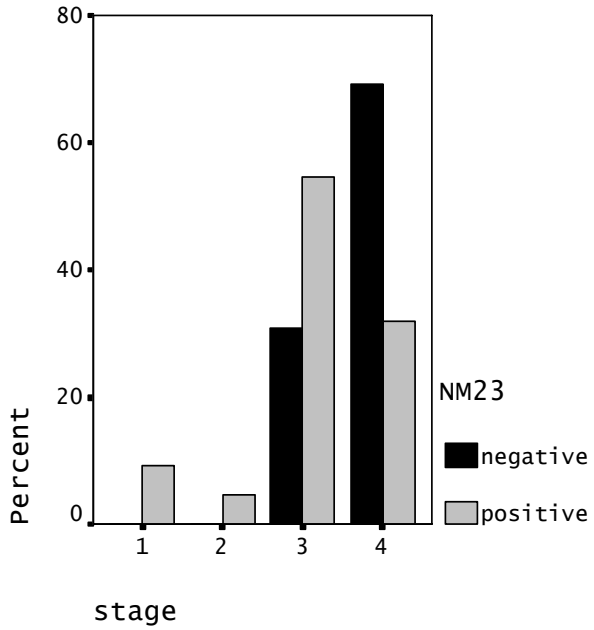

Figure 2. Relationship of stage and nm23 expression.

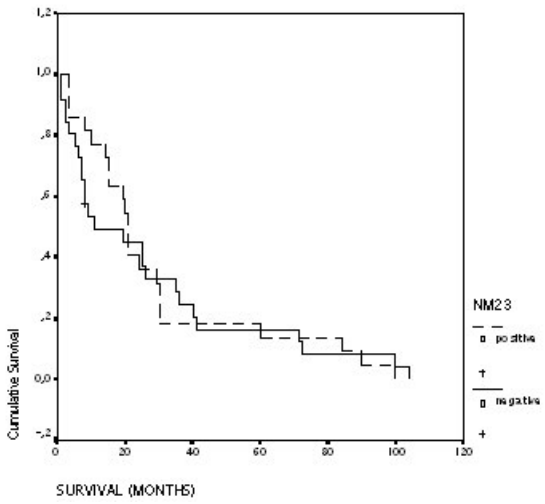

Figure 3. Survival curves of nm23 positive and negative cases according to overall survival.

markers that should be analyzed for each newly diagnosed patient. This list will be refined over time based on prospective evaluation of additional variables postulated to be of clinical utility.[3] Treatment of neuroblastoma depends on risk categorization system.

The most important variables predictive of disease outcome are age, stage at diagnosis, tumor site,

Table 1. Properties of the cases according to nm23 status

		N	Mean age (month)	Mean overall survival (month)	Favorable histology (n)	Stage* (%)	Sex (%) (M:male F:female)	Prognosis (%)	Chemotherapy response (%)
Nm23	+	26	36	29.55	32	I:9 II:4.5 III: 54.5 IV:32	M: 54.5 F: 45.5	Ex: 50 Alive :50	Good :68.2 Poor : 31.8
	–	22	33.8	25.85	27	I:- II:- III: 31 IV: 69	M: 50 F: 50	Ex: 42.3 Alive:57.7	Good : 65.4 Poor : 34.6

*p=0.006

myc N amplification, pathologic classification, serum ferritin, NSE, LDH levels, loss of heterozigocity, unbalanced gain of 17q, and expression of neurotrophin receptor TRKA.[3] There are still some cases with unexpected biologic behavior after treatment based on risk category system. New prognostic factors are required in management of neuroblastoma, to have longer survival rates, to overcome overtreatment and to prevent tumor split. The attempt to further identify the patients at high risk of metastases is very necessary to avoid unnecessary therapeutic procedures.

Nm23 gene expression in neuroblastoma is generally studied genetically on cell lines.[13,14] Nm23-H1 gene is localized in the region of 17q. The most common chromosomal changes in neuroblastoma are observed in this region.[19] Dr-nm23 is the third member of human nm23 gene family found to be associated with differentiation in neuroblastoma cells.[13,14]

It is suggested that biological significance of nm23 expression might be different in different tissues and neoplasms.[9] Nm23-H1 is known as a differentiation inhibitory factor. Plasma levels of nm23-H1 can also be measured by ELISA method. In myelodysplastic syndrome, nm23-H1 level was low in patients with low international prognostic scoring system.[20] This suggests nm23-H1 may be useful as a prognostic marker for MDS, especially in low risk patients. Immunohistochemical expression of nm23 was found more intensive inpatients with nonrecurring disease among 50 ovarian cancer patients.[8] In primary non-small cell lung cancer, nm23 is found to be a suppressor of systemic but not lymphatic metastasis.[21] In breast cancer, the expression of NDP kinase/ nm23 has been reported to correlate with good prognosis and a lack of nodal metastasis.[11] But, in retinoblastoma nm23 staining indicated a tendency to metastases.[12] In thyroid follicular carcinoma, a significant inverse association was observed between metastatic disease and nm23 H1 expression.[9] Nm23 H1 expression was related with tumor progression in nasopharyngeal carcinoma.[6] In rectal cancer, nm23 expression failed to correlate with distant metastasis.[5] In vitro transfection experiments showed that the nm23 gene suppresses metastasis, although the evidence from clinical studies was contradictory.[21]

Predicting the focus for investigating the metastatic potential of tumors is somewhat controversial. Should we examine the probable factors in the primary tumor or metastatic focus? The metastatic tumor cells might sometimes change some of their phenotypic properties. In this study we looked for nm23 expression in primary tumors that were obtained by thru-cut biopsies or primary resection materials. We did not include specimens from metastasis, recurrences or resected materials after treatment, because this study was designed to elucidate the usefulness of searching nm23 expression on primary tumors.

According to INSS, neuroblastomas are localized tumors in stage 1 and 2. Stage 3 cases are unresectable tumors with or without regional lymph node involvement. However, stage 4 or 4S includes cases with distant dissemination.[3] In this study we compared stage 1, 2 and 3 cases with stage 4 cases to understand the role of nm23 expression on metastasis. In our series, nm23 positive cases were more common in stage I, II and III groups. There were nm23 positive cases in stage IV cases, but they were usually in the older age group.

The rate of stage IV disease (metastatic) was 31.8% in nm23 positive cases and 69.2% in negative cases. Thus nm23 protein immunoreactivity was associated with the metastatic potential but mortality rates did not differ. The reason why we failed to demonstrate a correlation between the expression of nm23 and prognosis might be the shorter follow-up time of our resent cases.

We conclude that, negativity of nm23 expression might be a predictor for metastasis in neuroblastomas but it is not an independent prognostic factor. Prognosis in neuroblastoma is multifactorial. Nm23 expression indirectly affects prognosis, predicting patients at risk for metastasis.

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