Alternative prognostic factors in pediatric embryonal rhabdomyosarcoma: Nm23 expression, proliferative activity and angiogenesis

Gülden Diniz, Safiye Aktaş, Ragıp Ortaç, Ayşe Erbay, Canan Vergin, Malik Ergin

Dr. Behçet Uz Children's State Hospital, İzmir, Turkey

SUMMARY: Diniz G, Aktaş S, Ortaç R, Erbay A, Vergin C, Ergin M. Alternative prognostic factors in pediatric embryonal rhabdomyosarcoma: Nm23 expression, proliferative activity and angiogenesis. Turk J Pediatr 2004; 46: 239-244.

Possible clinical relevance of Nm23 expression, angiogenesis and proliferative activity were evaluated as prognostic parameters in childhood embryonal rhabdomyosarcoma (RMS).

Specimens of 25 RMS cases were studied for Nm23 antigen immunohistochemically. Vascular surface density (VSD) and number of vessels per stroma (NVES) calculated by stereologic methods on labeling sections with CD34 antibody. For evaluation of proliferative activity of tumors, mitotic figures and Ki67 positive cells were investigated. All findings were searched statistically.

Five patients were stage 1 (20%), two were stage 2 (8%), 15 were stage 3 (60%) and three were stage 4 (12%). The mean event free survival (EFS) was 20.8 and the mean overall survival (OS) was 25.9 months. Sixteen patients (64%) were alive and without disease. The percentage of Nm23 positivity was 52%. Log rank analysis showed Nm23 as a predictor for survival (p=0.0313). In Pearson correlation analysis, there was statistical significance between OS and presence of Nm23 expression (p=0.044). VSD was also positively related with EFS (p=0.040).

Despite the present parameters in use, there is a need for new prognostic markers, especially to predict the outcome of patients. These findings suggested that Nm23 expression and VSD might be useful for follow-up in RMS.

Key words: rhabdomyosarcoma, Nm23, Ki-67, angiogenesis, mitosis.

Rhabdomyosarcoma (RMS) is the most common pediatric sarcoma, accounting for about 50% of childhood malignant soft tissue tumors[1]. The natural course of the tumor is extensive local recurrence with metastases to lung, lymph nodes, liver and bone[2,3]. In the past, the five-year survival was less than 20%. Recently, therapeutic advances have dramatically improved the prognosis of patients with RMS. Individual patient survival and determination of treatment depend primarily on three factors: the histological subtype, the primary site of the neoplasm and the stage[4-5]. Despite the existing clinical parameters in use, there is a need for new prognostic markers, especially for use during the initial diagnostic period, for planning the minimum but most effective therapy and for predicting the outcome of treatment.

The evaluation of the degree of cellular proliferation in tumor tissue is the most useful means to predict the behavior of tumors[6]. The old but still widely used method is mitotic count in routinely processed sections. Cell proliferation can also be investigated with immunohistochemical staining for nuclear antigens related to cell growth and division such as Ki-67, Ki-S1 and PCNA[6,7]. Similarly, evaluation of the tumor's vessel number and density is another indicator of prognosis[7]. A growing body of literature has appeared in the past few years concerning the potential importance of determination of vascularization in tumor tissue[8,9].

Nm23 gene family is well known to include putative metastasis suppressor genes which are related with differentiation. Four types are

defined. Nm23-H1, Nm23-H2, Dr-Nm23 and Nm23-H4 genes encode nucleoside diphosphate (NDP) kinase[10]. These genes are expressed in different tumor types where their levels are alternatively associated with reduced or increased metastatic progressive potential, such as in neuroblastoma[10], rectal cancer[11], nasopharyngeal carcinoma[8], serous ovarian carcinoma[12,13], thyroid carcinoma[14], lung cancer[15], breast cancer[7] and retinoblastoma[16].

The aim of the present research was to determine Nm23 expression, percentage of Ki-67 labelling, vascularization and mitosis in the tumor tissue of 25 RMS cases and to correlate these findings with other prognostic factors.

Material and Methods

Twenty-five pediatric patients with embryonal RMS were included in this study. All patients were diagnosed and followed in the Children's Education and Research Hospital by the Oncology Study Group between 1991 and 2001. They were treated by one or more of surgery, chemotherapy and radiotherapy according to individual features[3]. All patients were treated with the International Society of Pediatric Oncology (SIOP) malignant mesenchymal tumor (MMT)'89 protocol[4].

Individual patient database was reviewed in all cases. Stage was assigned for patients based on primary tumor site, tumor size, tumor invasiveness, regional lymph node involvement and metastases. In addition, age, sex, histological subtype, surgical procedure, event-free (EF) and overall-survival (OS) were investigated (Table I).

All tumor tissues were obtained before chemotherapy as tru-cut or surgical biopsy specimens. Formalin-fixed and paraffin-embedded, well-preserved tissue blocks of tumors were used for immunohistochemical

Table I. Patient Characteristics and Status of Nm23, Ki-67 Mitosis and Vascularization for All Patients

Patient No.	Age (year)	Sex (M/F)	Histology (1)	Site (2)	Size (3)	Stage	Surgery (4)	EFS (months)	Os (months)	Outcome (5)	Nm23	Ki-67 (%)	VSD (mm^3)	NVES (mm^2)	Mitosis/10hpf
1	2	M	A	2	L	3	I	3	3	D	+	10	5.3	10.1	42
2	9	M	B	2	L	4	T	98	98	A	+	30	10.7	9.3	3
3	2	M	N	2	L	3	I	94	94	A	+	35	30.8	39.0	14
4	4	F	B	2	L	3	T	25	48	D	+	15	15.3	4.9	4
5	13	M	N	2	S	2	T	8	72	A	+	7	14.5	17.7	20
6	1	M	N	2	L	4	N	1	1	D	–	15	18.3	8.4	8
7	2	M	A	2	L	3	T	60	60	A	+	10	11.9	6.9	16
8	3	M	N	1	S	1	T	53	53	A	–	25	19.0	16.5	18
9	7	M	N	1	L	3	I	1	1	D	–	5	10.3	5.6	40
10	14	M	N	1	S	1	T	50	50	A	+	20	13.6	7.0	8
11	4	M	N	2	L	3	N	5	5	D	+	40	25.0	27.6	35
12	3	F	N	2	L	3	T	2	6	D	–	5	7.8	4.7	15
13	3	M	N	2	L	3	T	24	36	A	–	5	10.3	7.8	7
14	2	M	N	2	L	3	T	19	19	A	+	20	7.6	8.4	6
15	7	M	B	1	S	1	I	9	15	A	–	10	13.9	11.6	2
16	11	F	N	2	L	3	N	3	3	D	–	4	9.1	8.4	4
17	2	F	N	1	S	1	T	12	12	A	+	40	9.9	16.0	6
18	15	M	A	2	L	4	N	1	12	D	–	16	5.3	4.2	24
19	14	M	N	1	L	2	T	7	14	D	–	8	9.9	10.1	2
20	4	M	N	1	L	3	I	10	10	A	–	5	10.0	10.2	6
21	16	M	S	1	L	3	I	12	12	A	+	13	15.5	10.6	18
22	2	F	A	2	L	3	I	9	9	A	–	25	9.5	6.9	10
23	5	M	S	1	L	3	I	7	7	A	+	33	14.4	19.3	14
24	8	F	N	1	L	3	N	4	4	A	–	15	14.7	18.6	30
25	8	F	N	1	S	1	N	3	3	A	+	55	10.6	9.6	4

1. Classification of embryonal rhabdomyosarcoma: (B) (botryoid), S (spindle), A (anaplastic), and N (NOS).
2. Anatomical location of tumors was scored as 1-2, where 1=orbit, head and neck (nonparameningeal), genitourinary (nonbladder-nonprostate) and 2=other sites.
3. S (small)=tumor was 5 cm or less in diameter, L (large) tumor diameter was greater than 5 cm.
4. Surgical treatment was symbolized as T (total excision), I (incomplete resection) and N (no surgery, only performed biopsy).
5. Outcome of patients=A (alive) or D (died of disease).

VSD : vascular surface density.
NVES: Number of vessels per stroma.
EFS : event-free survival.
OS : overall survival.
NOS: not otherwise specified.

(IHC) study. We used negative control for rabbit primary antibodies (DAKO, N1699, USA) in Nm23 and Ki67 immunohistochemical staining.

Immunohistochemistry (IHC) was performed by streptavidin-biotin peroxidase method. Nm23-NDP kinase Ab-1 (1/25 diluted, Neomarkers, USA), Ki-67 (DAKO, USA) and CD34-Ab1 (1/25 diluted, Neomarkers, USA) were applied as primary antibodies. The first is a rabbit polyclonal antibody that recognizes the products of Nm23-H1 and H2 (the epitope is aa 86-102; molecular weight of the antigen is 17kDa and 185kDa). Invasive ductal carcinoma of breast was used as positive control for Nm23 staining, and lymphoid tissue was used for Ki67.

The evaluation was made with no knowledge of any of the clinical features. Diffuse or focal cytoplasmic staining for Nm23 was considered as positive (Fig. 1). Thus, the Nm23 expression was graded as negative and positive. The

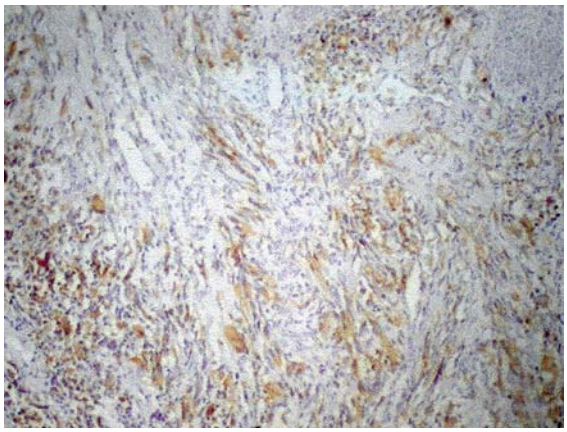

Fig. 1. Nm23 cytoplasmic expression in many rhabdomyoblasts (DAB x 100).

immune staining for Ki-67 was scored according to intensity and quantity. On each slide, a minimum of 1000 tumor cells were observed and positive cells were counted. The degree of positive cells was classified as low or high according to a calculated cut-off value. This value was found as 150/1000 for Ki-67 on SPSS program with roc curve.

The CD34 labelling slides were investigated for the evaluation of vessel status. The most vascularized areas were chosen for assessment at x40 magnification. An ocular square lattice with 121 points composed of 11 horizontal and 11 vertical test lines with known total test line length (LR=2.6875 μm) was superimposed on the test fields. The number of intersections (In) between the test lines and the labeled vessel walls was counted The area of each measuring field was 0.36 mm^2. The points without vessel touch were counted (Istr). Volume portion of stroma Vv (str) was computed according to Vv (str=Istr/121, and vascular surface density (VSD) was computed according to VSD = [In x 2/LR x Vv (str)]. The number of vessels (N) within the measuring field was counted and the number of vessels per mm^2 stroma (NVES) was computed according to NVES = N/Vv. The VSD and NVES values of each patient were calculated by this method[17-18] and the cut-off values were found on SPSS. These values were 10.4/mm^3 for VSD and 10.1/mm^2 for NVES.

Mitotic figures were investigated in section staining with hematoxylene-eosin. The number of mitoses of 10 consecutive high-power fields was counted.

Pearson correlation analysis, linear regression 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 were considered to be statistically significant. Descriptive and frequencies of the parameters were evaluated.

Results

The mean age of the patients was 6.4±4.87 years. Eighteen were male (72%) and seven were female (28%). As is shown in Table II, according to the

Table II. Properties of the Cases According to Patient Status

Patient status	Histological subtype of tumor	Percent %	Stage	Percent %	Surgical procedure	Percent %
Died of disease	Anaplastic	22.2	I	0	No surgery	44.4
	NOS	66.7	II	11.1	Incomplete	22.2
	Botryoid/spindle	11.1	III	66.7	recection	
			IV	22.2	Total excision	33.3
Alive	Anaplastic	12.5	I	31.3	No surgery	12.5
	NOS	62.5	II	6.3	Incomplete	37.5
	Botryoid/spindle	25	III	56.3	resection	
			IV	6.3	Total excision	50

modified IRSG[4] classification five, cases (20%) were botryoid and spindle cell variants (which were emphasized to have a superior prognosis), 17 cases (68%) were conventional embryonal RMS (NOS) and three cases (12%) anaplastic variant. Similarly, 11 tumors (44%) had a good prognostic location, while 14 (56%) had poor location, Six tumors (24%) were small (5 cm or less diameter) and 19 tumors (76%) were large (greater than 5 cm). Five patients were stage 1 (20%), two were stage 2 (8%), 15 were stage 3 (60%) and three were stage 4 (12%). Clear surgical margins were obtained in only six (24%) patients. Eighteen patients had no second operation and nine of them were evaluated as inoperable. The cases had been on clinical follow-up for 1-98 months (mean: 34.6 months). The mean EFS was 20.8 and the mean OS was 25.9 months. Sixteen patients (64%) were alive and without disease. The percentage of Nm23 positivity was 52%. The mean mitotic figures were 14.24/10hpf. Ki67 labelling was found higher than cut-off values in 14 cases (56%). Similarly, 12 cases (48%) had high VSD and 12 cases (48%) had high NVES degree (Table III).

Log-rank analysis (Fig. 2) showed Nm23 as a predictor for survival (p=0.0313). In Pearson correlation analysis, there was statistical significance between Os and presence of Nm23 expression (p=0.044). A positive relationship was established between VSD and EFS (p=0.040) and a negative relationship between "disease-related death" and size of tumor (p=0.036). None of the newly examined parameters were independent factors for stage or survival on linear regression analyses.

Table III. Prognostic Parameters in Tumors According to Patient Status

	Ki-67	VSD	NVES	Mitosis
Died of disease	13.11	11.81	9.33	19.33
Alive	21.75	13.55	13.46	11.37
Total	18.64	12.92	11.97	14.24
P	0.506	0.291	0.974	0.982

VSD: vascular surface density; NVES: number of vessels per stroma.

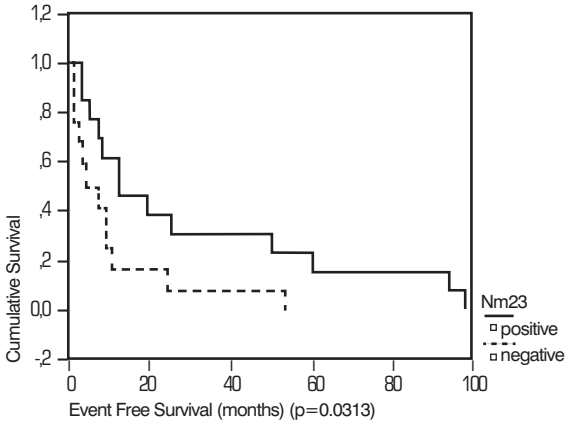

Fig. 2. Cumulative event-free survival curves for Nm23 status in pediatric rhabdomyosarcomas.

Discussion

There are many prognostic factors considered for almost all neoplasms. These markers 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. In addition, identification of prognostic factors and the use of risk-directed multimodal therapy have improved the outcome for these patients. A risk categorization system for RMS was developed by IRSG[5]. The most important variables for evaluating disease outcome are histologic subtype, location of tumor and stage. But there are still some cases with unexpected biologic behavior after treatment based on these parameters. New prognostic factors are required in the management of RMS. The attempt to further identify patients at high risk to develop metastases of tumors is crucial to avoid unnecessary therapeutic procedures[1,4].

With development of genetic science, several gene changes in tumors have been explored. Most studies have indicated that some genetic fusion, amplification and translocation are associated with biologic behavior of the tumor. Recognition of specific genetic changes in a malignancy has allowed better understanding of the pathogenesis of this disease[6]. The most investigated parameters in RMS are PAX3-FKHR and PAX7-FKHR gene fusion[19,20], MYCN amplification[21], MAGE-BAGE-GAGE gene expressions[22] and ccn3 (Nov) gene alteration[23]. The Nm23 gene family is also known to associate with metastasis and differentiation. Prognostic importance of Nm23 expression has been widely investigated for several nekoplasms[7-16]. On the contrary, there have been only a few studies investigating the association between Nm23 gene expression and survival in patients with sarcomas[24].

It has been suggested that biological significance of Nm23 expression may vary in different tissues and neoplasms. For example, Dr-Nm23, the third member of the human

Nm23 gene family, is found to be associated with differentiation in neuroblastoma cells[10]. Immunohistochemical expression of Nm23 was found more intensive in patients with nonrecurring disease and living patients among 50 ovarian cancer patients[12]. In primary, non-small cell lung cancer Nm23 is found to be a suppressor of systemic but not lymphatic metastasis[15]. In breast cancer[7], the expression of NDP kinase/Nm23 has been reported to correlate with good prognosis and a lack of nodal metastasis. But in retinoblastoma[16] Nm23 staining was observed to indicate a tendency to metastases. In thyroid follicular carcinoma[14], a significant inverse association was observed between metastatic disease and Nm23 H1 expression. Nm23 H1 expression was related with tumor progression in nasopharyngeal carcinoma[8]. In rectal cancer Nm23 expression failed to correlate with distant metastasis[11]. In vitro transfection experiments show that the Nm23 gene suppresses metastasis, although the evidence from clinical studies is contradictory.

The development of intensive multidisciplinary treatment protocols has resulted in significant improvements in outcome of RMS, especially for patients with local or locally extensive disease. For these patients a 60%-70% EFS can be expected. Despite an aggressive approach, the outcome for patients with metastatic disease remains poor[1-5]. The evaluation of metastatic potential of a tumor is very important. Because most of our RMS patients died with distant metastasis, we hypothesized that alteration of Nm23 expression can be an indication of metastatic disease. Although a large prospective study is needed to provide definitive conclusions, our findings suggest that Nm23 expression might be a prognostic factor for better outcome in RMS.

Similarly, evaluation of the tumor's vessel number and density is another prognostic indicator for most tumors. Vascularization and its contribution to tumor growth have been widely studied in different neoplasms. A growing body of literature has appeared in the past few years concerning the potential importance of determination of vascularization in tumor tissue[9,17,18]. In some of these studies, intratumoral microvessel density was found to have prognostic value for survival, because the new capillary network is required for growth of the solid tumor. The term angiogenesis was coined in 1935 to describe the formation of new blood vessels in the placenta[9]. The reason why we chose stereologic assessment of vascularization by VSD and NVES was to avoid methodological disadvantages of quantification of vascularization caused by two dimensional sections or errors that might be caused by observer bias. This study suggests that quantitative assessment of vascularization, especially measurement of VSD, might be useful for evaluation of survival in RMS.

Both nuclear morphometry and evaluation of the cell proliferative activity have been reported to be useful tools in predicting prognosis in malignant tumors. Cellular features, especially DNA ploidy and S-phase fraction, that can be evaluated with flow cytometry definitely correlate with prognosis. In addition, several other methods are available to determine the degree of cellular proliferation in tumor tissue. The old but still widely used method is mitotic count in routinely processed sections. Cell proliferation can also be investigated with immunohistochemical staining for nuclear antigens related to cell growth and division such as Ki-67, Ki-S1 and PCNA. Nuclear Organizer Region (NOR) evaluation with AgNOR staining is also another indicator of cell proliferation[6].

Most studies have indicated that mitotic count is not useful for prediction of prognosis in RMS because high mitotic rates are uncommon especially in well differentiated subtypes. Up to 20-30 mitoses per 10 high-power fields were only found in anaplastic or alveolar variants[2]. Similarly, there is no obvious relevance with Ki-67 labelling[7,25,26] and prognosis in RMS in the literature. There have been few reports on RMS regarding cellular proliferation and the findings of these studies are contradictory. The present study also suggests that mitotic degree and Ki-67 expression are not predictive for prognosis in RMS and this result correlates with other studies.

We conclude that Nm23 expression and VSD number might be predictors for metastasis in RMS, but they are not independent prognostic factors. Prognosis is affected multifactorially. Nm23 expression and VSD number indirectly affect prognosis-predicting patients at risk to develop metastasis. These biologic markers show promise for RMS, but this study is with a limited number of cases in one center. Finally, identifications of alternative prognostic markers specific for RMS will

help us better understand the mechanisms involved in the pathogenesis and facilitate the development of novel effective therapies.

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