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Original Article

Role of NELL2 and LAMA2 immunohistochemistry in the histological classification of ependymomas in adult and pediatric patients

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Abstract

Aim: Ependymomas are highly heterogeneous neuroepithelial tumors that occur in all age groups and in all compartments of the central nervous system. Because of the tumor heterogeneity, the current histopathological criteria for grading are insufficient. Therefore, auxiliary prognostic markers that are cost-effective and easy-to-use and may help predict clinical behavior are still needed. Neural Epidermal Growth Factor Like Like-2 (NELL2) and laminin alpha-2 (LAMA2) are two outstanding proteins with prognostic significance in ependymomas. In the present study, we aimed to investigate the correlation of NELL2 and LAMA2 expression with histopathological parameters in patients with ependymomas.

Materials and Methods: A total of 64 ependymomas were retrospectively evaluated and representative paraffin blocks were selected. Demographic and histopathological characteristics of the patients were noted. Slides from selected blocks were immunohistochemically stained using NELL2, LAMA2 and Ki67 antibodies. The results were compared with other characteristics of the patients.

Results: We found that the histological grades of the ependymomas correlated with age, tumor localization, histological subtype and number of mitosis. NELL2 staining was significantly higher in grade II ependymomas, while LAMA2 staining was significantly higher in grade III ependymomas. Moreover NELL2 and LAMA2 expression was associated only with the histological grade of the tumor. We found the Ki-67 proliferation index to be consistent with increasing histological grade.

Conclusions: Our findings show that higher NELL2 expression than LAMA2 expression is associated with low histological grade and probably better prognosis. On the other hand LAMA2 expression is associated with high histological grade and probably poor prognosis.

Keywords: Ependymoma; brain tumor; Ki67; LAMA2; NELL2

INTRODUCTION

Ependymomas arising from ependymal cells of the ventricular system and spinal canal, account for 6.8% of neuroepithelial tumors. They can occur in all compartments of the central nervous system and at any time in life, with two major peaks around the age of 0-4 and 30-40 years (1,2). Ependymal neoplasms most frequently occur in the spinal canal (46%) of adult and in the posterior fossa (80%) of children aged under 3 years (3,4). Ependymomas represent a highly heterogeneous group of tumors because of their wide age distribution and their ability to have different localizations (2). At present, total surgical excision is the standard treatment for all ependymomas. Definitive or adjuvant radiotherapy is performed in cases in which surgical treatment is not appropriate. The 5-year survival rate of patients with ependymomas ranges from 30% to 60%. Pediatric ependymomas have a worse prognosis than adult ependymomas, with a 5-year survival rate of 42.4% (5). Histological grading is the basic criterion for determining the biological behavior of ependymomas and for making clinical treatment choices. Ependymomas are histopathologically graded according to the World Health Organization (WHO) recommendations based on necrosis, mitosis, microvascular proliferation, and the Ki-67 proliferation index. However, only histological evaluation is not sufficient to predict the clinical course of the patients, because different subtypes of ependymomas with the same histological grade may exhibit different

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clinical behaviors (6). The prognostic markers known to predict the clinical behavior of ependymomas are rather limited and new markers are still needed. Neural epidermal growth factor-like like-2 (NELL2) is a protein that plays a role in neural development and differentiation. However, its expression profile and role in cancer cells remain unclear (7). The laminin alpha-2 (LAMA2) chain of the LAMA subunit is the major component of the basal membrane of the gliovascular unit formed by astrocytes, pericytes and vascular smooth muscle cells. It is necessary for vascular development and its deficiency has been shown to cause various defects (8,9). Recent studies have indicated that NELL2 and LAMA2 may play important role in grading pediatric posterior fossa ependymomas (PF-EPNs) and predicting the prognosis of patients: however, no study has confirmed this so far (10,11).

In the present study, we aimed to re-evaluate all subtypes of ependymomas with different histological grades in terms of known prognostic factors and to assess the correlation of NELL2 and LAMA2 expression with these parameters.

MATERIALS and METHODS

In the present study, the data of 64 patients who underwent surgery in our hospital for ependymomas between 2002-2015 were retrospectively evaluated. Demographic data such as age, sex, and tumor location of all the patients were noted. Hematoxylin and eosin stained slides obtained from formalin-fixed paraffin-embedded tissues of the patients were re-examined under the Olympus CX31 light microscope with a standard 22-mm-diameter eyepiece. The histological type, histological grade, number of mitosis, presence of necrosis, presence of vascular endothelial proliferation and Ki-67 proliferation indeces were noted (2). In addition, recurrence and survival data were obtained from the patients' medical records.

The ependymomas were divided into three groups based on their histological grades, in accordance with the WHO classification of central nervous system tumors recommendations (2). Immunohistochemical staining was performed using Ki67 (MIB1 clone (Biogen, 1/100 dilution rate), NELL2 (Abcam, catalog number: ab80885, 1/200 dilution rate) and LAMA2 (Abcam, catalog number: ab55409, 1/500 dilution rate) antibodies by taking 3-5 µm sections from the areas of paraffin blocks that best reflected the tumor. In accordance with the required conditions for staining, the sections were incubated overnight at 56°C after deparaffinization, immunohistochemical staining was performed in a Leica Bond-Max automatic immunostainer according to the manufacturer's protocols. Brain tissue and placental tissue were used as positive controls for NELL2 and LAMA2 antibodies, respectively.

In each case, 1000 tumor cells were counted and the Ki-67 nuclear staining percentage was calculated. The results were noted as the Ki-67 index. NELL2 and LAMA2 expression was evaluated according to the cytoplasmic staining of tumor cells. For LAMA2 expression membranous positivity was also included into the evaluation.

The staining intensity was scored from 0 to 3 and was interpreted as follows: score 0, no staining of tumor cells, : score 1, mild severe staining: score 2, moderate staining, and score 3, strong staining.

The staining distribution was scored from 0 to 3 by evaluating the percentage of stained tumor area on the slide and was interpreted as follows: score 0 staining between 0% and 1%: score 1, focal staining between 2% and 35%: score 2 ,moderate staining between 36% and 70% , and score 3, diffuse staining between 71% and-100%.

The immunohistochemical score (IS) is calculated by summing the intensity and distribution scores and again rated 0-3 as shown in Table 1. The patients with total IS 0 and 1 were considered as negative, and IS 2 and 3 were considered as positive.

In the tumor samples the Ki67, NELL2 and LAMA2 expressions of the patients were compared with demographic and histopathological data for prognostic significance.

Table 1. Criteria used in immunohistochemical evaluation

	IS (Intensity + distribution of the staining)	
Negative	IS 0	0
	IS 1	1 and 2
Positive	IS 2	3 and 4
	IS 3	5 and 6

IS: Immunohistochemical score

Statistical Analysis

Statistical analysis was performed by using the "SPSS for Windows Version 15.0" package program. Numerical variables were summarized with mean ± standard deviation and categorical variables were summarized by number and percentage.

The significance of the difference between the two groups in terms of averages was investigated by t test. The significance of the median values between the two groups was investigated via the Mann Whitney test. ANOVA variance analysis test was used to investigate the significance of differences between more than two groups averages. To test the significance of median values between more than two groups Kruskal Wallis test was used. Categorical variables were assessed by Pearson Chi-Square or Fisher exact test.

Relationship between continuous variables was investigated by Spearman correlation test. The significance level was determined as $p< 0.001$.

RESULTS

In the present study, we evaluated both NELL2 and LAMA2 expressions in 64 ependymomas samples by IHC. Besides, we examined the association of those protein expressions with histopathological and prognostic parameters of tumors.

Over 64 patients 35 were male and 29 were female. The ages were distributed in a wide range from 5 months to 73 years. 53.1% of the tumors were located in the spinal cord (n= 34), 37.3% in the supratentorial region (n = 24) and 9.4% tumors in the posterior fossa (n= 6). A total of 9 cases were under 14 years of age and all tumors in pediatric patients were located in the posterior fossa. The grate majority of the tumors in the adult age group was located the spinal cord. 56.3% of the tumors were classical ependymomas (n = 36), 21.9% of them were anaplastic ependymomas (n= 14), 15.6% of them were myxopapillary ependymomas (n= 10) and 6.2% of them were subependymomas (n= 4) according to WHO histopathological classification.. 21.9% of the cases were grade I (n=14), 56.3% of the cases were grade II (n= 36) and 21.9% of the cases were grade III (n=14) (Table 2).

The gender distribution among histological grades of ependymomas was similar (p= 0.534). We found significant differences in age, tumor localization, and the number of mitosis in 10 high-power-field with different histological grades (p< 0.001) (Table 2).

Average Ki-67 proliferation index in patients was 5.6% (max:50%, min:1%). The differences between the Ki-67 proliferation index and tumor grade were statistically significant(p< 0.001) (Table 2).

Among 64 cases, 7 were recurrent. 7.1% (n= 1) ingrade I ependymomas, 8.3% (n= 3) in grade II ependymomas, and 21.4% (n= 3) in grade III ependymomas. However the relation between recurrence and tumor grade was not statistically meaningful (p> 0.05) (Table 2).

NELL2 expression was observed in 14.3% of grade I ependymomas but this was not statistically meaningful. (Table 3). 61.1% of the grade II ependymomas showed positive staining with NELL2 and 50% of these cases were IS 2 (Figure 1). Only 7.1% of the grade III ependymomas expressed at a low level of NELL2; but this was not significant. We observed significant differences between the grade II-grade III ependymomas and also between the grade II -grade I ependymomas regarding NELL2 protein expression by the statistical analysis. (p< 0.001) (Table 3).

Table 2. Distribution of the demographical and histopathological data of the cases

	Ependymoma				P value
	Grade I (n=14)	Grade II (n=36)	Grade III (n=14)	Total (n=64)	
Age (min-max)	35.86 (22-53)	42.15 (3-73)	22.37 (5ay- 68y)		P<0.005*
Gender					
Male	9 (64.3%)	19 (52.7%)	7 (50%)	35 (54.7%)	0.534
Female	5 (35.7%)	17 (47.3%)	7 (50%)	29 (45.3%)	
Localization					
Spinal cord	10 (71.4%)	21 (61.1%)	3 (21.4%)	34 (53.1%)	
Supratentorial	4 (28.6%)	13 (33.3%)	7 (50%)	24 (37.5%)	p<0.001*
Cerebellum		2 (5.6%)	4 (28.6%)	6 (9.4%)	
Histological subtype	Myxopapillary (%15.6) Subependymoma (%6.2)	Classic (%56.3)	Anaplastic (21.9%)	64	P<0.001*
Mitosis count[a] (min-max)	0-1	1-2	2-6		p<0.001*
Ki-67 (%)	1.07%	3.36%	16%		p<0.001*
Recurrence					
present	1 (%7.1)	3 (%8.3)	3 (%21.4)	7 (%10.9)	p>0.05
absent	13 (%20.3)	33 (%51.7)	11 (%78.6)	57 (%89.1)	

[a]per 10 High power field ; *Statistically significant

Table 3. NELL2 expression in ependymoma cases

		Ependymoma		
		Grade I (n=14)	Grade II (n=36)	Grade III (n=14)
NELL2	Negative	IS 0: 12/14 (85.7%) IS 1: 2/14 (14.3%)	IS 0: 4/36 (11.1%) IS 1: 10/36 (27.8%)	IS 0: 13/14 (92.9%) IS 1: 1/14 (7.1%)
	Positive		IS 2: 18/36 (50%) IS 3: 4/36 (11.1%)	
P value		p>0.05	p<0.001*	p>0.05

IS: Immunohistochemical score

Table 4. LAMA2 expression in ependymoma cases				
		Ependymoma		
		Grade I (n=14)	Grade II (n=36)	Grade III (n=14)
LAMA2	Negative	IS 0: 6/14 (42.8%)	IS 0: 23/36 (63.9%)	IS 1: 1/14 (7.1%)
		IS 1: 8/14 (57.2%)	IS 1: 12/36 (33.3%)	
	Positive		IS 2: 1/36 (2.8%)	IS 2: 9/14 (64.3%)
				IS 3: 4/14 (28.6%)
P value		p>0.05	p>0.05	p<0.001*

IS: Immunonhistochemical score; *Statistically significant

All cases of grade I ependymomas were evaluated as negative for immunostaining with LAMA2. Only 2.8% of the grade II ependymomas were positive. However, 92.9% of grade III ependymomas showed LAMA2 positivity. A statistically significant difference was observed between grade II and grade III ependymomas concerning LAMA2 expression (p< 0.001).(Table 4) The basal membrane of vascular structures was also positively stained with LAMA2 (Figure 2).

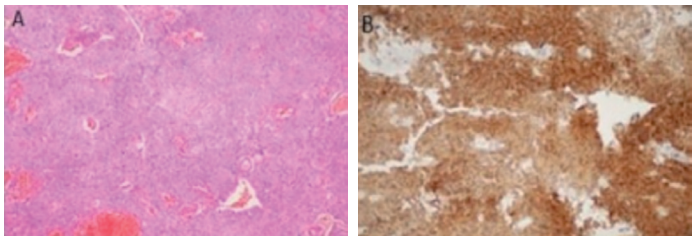

Figure 1. Histopathological appearance of grade II ependymoma; (a) Hematoxilen&Eosin staining (H&E, x100). (b) diffuse positive staining with NELL2 antibody (x200)

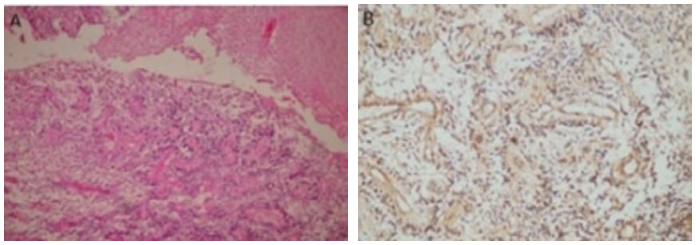

Figure 2. Histopathological appearance of grade III ependymoma; (a) Hematoxilen&Eosin staining (H&E, x100). (b) positive staining with LAMA2 antibody (x200)

NELL2 and LAMA2 expressions did not demonstrate any meaningful relation with histopathological parameters other then tumor grade. (Table 3 and Table 4) Likewise, we observed no correlation between clinical parameters and our immunohistochemical study results.

DISCUSSION

In the present study showed a positive correlation between the histological grades of ependymomas correlate well with age, tumor localization, histological subtype and the number of mitosis. We have also found that the Ki-67 proliferation index is positively correlated with the histological grade. Our study is also demonstrated that the staining of the NELL2 antibody in grade II ependymomas may help to distinguish those tumors from the other histological grades. LAMA2 antibody expression distinguishes grade III ependymomas from the other histological grades of ependymomas.

Ependymomas are glial tumors arising from ependymal cells and showing morphological, immunophenotopic and ultrastructural similarities to these cells that laid the cerebral ventricular system (1,12). The 25-40% of the ependymoma patients are younger than 2 years-old and the tumors of this period are mostly intracranial and especially infratentorial. The second most common localization is the spinal region and the tumors are presented around 40 years of age (6). Consistent with literaure,in our study the most common localization was infratentorium for pediatric patients and spinal region for adult patients over 30 years old.. Ependymomas are seen almost equally in men and women, both (1.77:1) in our study and the literatüre (13).

As it is known, there is no definite 'cut-off' value for the mitotic while the grading of ependymomas .However, Tihan et al. proposed that the tumors with less than five mitotic figures per 10 HPF should be regarded as grade II ependymoma. Besides, tumors with more than 10 mitotic figures per 10 HPF should be regarded as grade III ependymoma. Moreover the tumors with mitotic rates between 5 and 10 per 10 HPF should be graded according to the presence of additional criteria such as vascular proliferation and increased cellularity (14). In our study, there was only one grade III ependymoma case which has higher than 5 mitotic figures per 10 HPF. Therefore mitotic count alone didn't help us to grade tumors in this study, but we observed that mitotic count in 10 high power fields was related to the grade of ependymomas. (Table 2)

Although the Ki-67 index is of prognostic value in many tumors, for ependymomas, there are conflicting data in the literature. Zawrocki et al, showed the correlation of the high Ki-67 index with increased histological grade and poor prognosis (15). Similarly, we also found a significant correlation between the Ki-67 index and the increased histological grade in our study. (Table 2) However, Prayson et al. reported that Ki 67 index could not use to predict the biological behavior and other parameters like mitotic figure, viability, vascular proliferation and necrosis are more helpful in determining aggressive behavior (16,17).

Multiple factors, such as histological grade, tumor localization, age, sex, and tumor excision margins, have effects on recurrence potential of ependymomas (13). A high risk of recurrence was reported in incomplete resections, in pediatric tumors and anaplastic subtypes of ependymoma (18). In the present study, there were only 7 recurrent cases. The highest recurrence rate was in grade III anaplastic ependymomas with 21.4% (Table 2). Consistent with the literature, we observed that the incidence of recurrence is related to the high histological grade. However, because of limited number of patients, we could not show the statistical significance of this relationship. We didn't find any relationship between the risk of recurrence and other parameters such as age and tumor localization.

NELL2 is a protein that plays a role in neural development and differentiation. The function role of NELL2 protein in the differentiation of neurons is not fully understood, and its role in cancer cells remain uncertain. NELL2 exhibits increased expression in benign prostatic hyperplasia, prostate, bladder and breast cancers, and Hodgkin's lymphoma. NELL2 expression decreases in pancreatic and renal cancers (7). Expression of NELL2 and NELL1 has been reported in especially nontumoral areas in renal cell carcinoma (19). In a study of central nervous system tumors, Maeda et al. reported NELL2 expression increased in neuroblastoma, medulloblastoma, central neurocytoma and some astrocytic tumors, and loss of expression in glioblastoma (20). In the present study, we observed that NELL 2 protein was expressed in a significant part of patients. Maeda et al. indicated that NELL2 could show different expression in astrocytic tumors with different histological grades. Likewise, we observed that NELL2 expression was significantly different in various histological grades of ependymomas. The embryonal and postembryonal distribution of NELL2 vary in different areas of the brain and this suggests that its expression may differ according to the tumor location (21). However, in the present study, we could not detect any significant difference between NELL 2 expression and the tumor localization.

The extracellular matrix proteins play a critical role in the biological behavior of brain tumors. Laminin is the most abundant glycoprotein in the basal membrane and it has binding domains for both extracellular matrix and cell-surface receptors (23,24). LAMA2 chain of the laminin alpha subunit is the major component of the basal membrane of the gliovascular unit formed by astrocytes, pericytes and vascular smooth muscle cells . It is a necessary component for vascular development in the brain and has been shown to cause various defects in its deficiency (8,9). In the present study, we examined LAMA2 expression in all histologic types of ependymoma and found a significant difference in histologic grade III tumors. LAMA2 positivity was cytoplasmic and membranous in tumor cells. On the other hand in most of our patients vascular structures had some degree of LAMA2 staining along their basal lamina. As in our study, Vitolo et al. also reported expression in the basal membrane of vascular structures as a result of LAMA2 staining in neuroendocrine tumors of the lung (24). This suggests that the LAMA2 chain may be effective in the development of vascular structures in both normal and neoplastic tissues. Moreover, it is thought to be important in cell proliferation and tumor growth and also to increase the potential of tumor metastasis by disrupting vascular permeability and is associated with poor prognosis. As reported by Leonoudakis et al., we also found that LAMA2 expression was significantly increased in the most aggressive group, grade III anaplastic ependymomas (25). This suggests that increased expression of LAMA2 may be related to a more aggressive course of ependymomas and increased risk of metastasis and recurrence with the prospect of positivity in vascular structures. However, we couldn't detect any significant correlation with LAMA2 expression in recurrent patients, in different age groups, or cases with different localization. We know that posterior fossa ependymomas are more aggressive in children but we did not find any significant correlation between the prevalence and severity of LAMA2 expression in terms of age and tumor localization in pediatric patients. We think that we can not achieve the expected results because of limited number of cases. Further studies with larger sample size should be performed to present better the correlation of NELL2 and LAMA2 expressions to the histological grades or the other prognostic parameters.

To our knowledge, there are a few studies in the literature evaluating the expression of NELL2 and LAMA2 in ependymomas. Witt et al. investigated the presence of the NELL2 and LAMA2 in PF-EPNs by molecular and immunohistochemical methods. They suggested that the expression of these proteins could predict patients' prognosis. Besides, they reported that LAMA2 expressed ependymomas are more aggressive, have greater metastatic and recurrence potentials and have lower survival rates rather than NELL2 expressed ones. Similar to this study, we found that NELL2 and LAMA2 protein staining is significantly expressed in grade II ependymomas and grade III ependymomas, respectively. However, our findings were independent of tumor localization. More recent studies suggest that NELL2 and LAMA2 expression in ependymomas may be crucial for diagnosis and follow-up, and extensive researches are needed in this regard (10,11). According to our literature search, we couldn't find any study evaluating NELL2 or LAMA2 expression in low grade ependymomas. In this respect, our research is the first study in the literature. At the same time in an current study we know that CD44 is a reliable and prognostic biomarker that similar positive expression in pediatric posterior fossa ependymoma like LAMA2. Furthermore negative relationship determined between with NELL2 and CD44/LAMA2 expression profile in this study (26). On the other hand Araki et al. didin't find any significant prognostic association between tenascin-C (TNC) and LAMA2 expression in their study.

They analyzed TNC expression and chromosome 1q status in three moleculer subgroups in PF-EPN. They found that tumors with combined 1q gain and TNC expression had worse outcome (27).

CONCLUSION

In conclusion, our study showed that histological grades of ependymomas show a positive correlation with other parameters such as age, localization, histological subtype, number of mitosis. Besides, increased NELL2 expression compared to the LAMA2 is assosiated with low histological grade and probably better prognosis. Morover, increased LAMA2 expression is assosiated with high histological grade and presumably poor prognosis in ependymomas.term studies with a large sample size are warranted to evaluate the factors acting on the length of treatment continuation and drug survival.

Competing Interests: The authors declare that they have no competing interest.

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Ethical Approval: This study was approved by Institutional Review Board of Diskapi Yildirim Beyazit Research and Training Hospital (230315.21/18).

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