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## MERLIN EXPRESSION IN PEDIATRIC ANAPLASTIC EPENDYMOMAS REAL TIME PCR STUDY

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□ *The most common genetic abnormalities of ependymomas involve the chromosome 22 where there is the oncosuppressor gene neurofibromin 2 (NF2). NF2 mutations are primarily encountered in spinal lesions. In contrast, NF2 alterations do not seem related to tumor grade. We studied the NF2 expression through a real-time polymerase chain reaction in 25 pediatric anaplastic ependymomas. We compared the NF2 expression in neoplastic and non-neoplastic tissues, in supratentorial and infratentorial ependymomas and in primitive and non-primitive tumors (recurrences and metastases). Statistical analysis did not prove significant differences. Our results suggest that NF2 alterations are not typical of intracranial anaplastic ependymomas.*

**Keywords** ependymoma, anaplastic, merlin, NF2, RT-PCR

### INTRODUCTION

Ependymomas account for 3–9% of neuroepithelial tumors and are the third most common brain tumor diagnosed in children after pilocytic astrocytoma and medulloblastoma. They arise from ependymal cells lining the ventricles and the spinal canal. Little is known of the etiopathogenesis of ependymomas. An increased incidence of ependymomas, particularly spinal ependymomas, has been reported in patients affected by neurofibromatosis type 2 (NF2) syndrome. Actually, the most common genetic abnormalities of ependymomas involve the chromosome 22 where there is the

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oncosuppressor gene neurofibromin 2 (NF2). NF2 gene mutations are responsible for NF2 syndrome, which is associated with a high incidence of tumors (i.e., schwannomas, meningiomas, and ependymomas) [10, 16, 19].

The NF2 gene encodes the production of merlin (also called schwannomin). Merlin is a member of the proteins 4.1 superfamily. Within the 4.1 superfamily, merlin shares the highest degree of homology with a subgroup of proteins including ezrin, radixin, and moesin (ERM proteins) that in polarized cells link the actin of the cytoskeleton to cell membrane glycoproteins. The similarity of merlin with the ERM proteins indicates that its function may be related to those of the ERM proteins: organization of membrane extensions and cell adhesion, membrane traffic, and cell signaling. Merlin is expressed in a variety of tissues during embryonic development and in adulthood. In the normal central nervous system, merlin is widely expressed particularly in glia and neurons [8, 22]. Several experimental data demonstrated that the merlin over-expression results in a significant decrease in cell proliferation, reversion of Ras-induced transformation, and reduced tumor formation in nude mice. On the contrary, the inactivation of the NF2 gene leads to the development of tumors through the loss of contact-dependent inhibition of growth. In addition to the tumors that are classically associated with NF2 syndrome, NF2 mutations may be also found in other tumors, i.e., mesotheliomas of the lung. Furthermore, merlin expression is reduced in human malignant gliomas [4–7, 11, 12,14, 17,23, 24].

Approximately 29–38% of ependymomas show monosomy or translocations on chromosome 22 19 [3]. Among ependymomas, NF2 mutations are primarily encountered in spinal lesions. In contrast, it has been assumed that NF2 alterations do not significantly vary with tumor grade [1, 2, 9, 13, 18,20, 21, 25].

In the present work, we studied the NF2 expression through a real-time polymerase chain reaction (RT-PCR) in a group of 25 pediatric intracranial anaplastic ependymomas. In addition, we compared the merlin expression in supratentorial and infratentorial ependymomas and in primitive and non-primitive tumors (recurrences or metastases).

## PATIENTS AND METHODS

### Patients

Twenty-five tissue specimens were obtained from 16 consecutive patients affected by anaplastic ependymomas surgically treated at the Anna Meyer Pediatric Hospital in which fresh tumoral tissue was available for the RT-PCR. Eight (50%) were from men and 8 (50%) were from women. The average age at the time of the first surgery at our institution was 7 years (range, 1–17). (Table 1) None of the patients had clinical evidence of NF2 syndrome.

**TABLE 1** Clinical Characteristics of the Patients

ID	Pz	Age	Sex	Tumor	
1	BM	11	M	SUPRATENTORIAL	RELAPSE
2		12			RELAPSE
3		12			RELAPSE
4	BA	5	M	SUPRATENTORIAL	RELAPSE
5		6			RELAPSE
6		7			RELAPSE
7		9			RELAPSE
8	CC	1	F	INFRATENTORIAL	PRIMITIVE
9	FaF	14	F	SUPRATENTORIAL	RELAPSE
10		15			RELAPSE
11		15			RELAPSE
12	FrF	4	F	INFRATENTORIAL	PRIMITIVE
13	LM	10	M	SUPRATENTORIAL	PRIMITIVE
14	MG	1	M	INFRATENTORIAL	PRIMITIVE
15		1			PRIMITIVE
16	MI	13	M	SUPRATENTORIAL	RELAPSE
17		13			RELAPSE
18	NT	16	M	SUPRATENTORIAL	RELAPSE
19	RE	5	F	INFRATENTORIAL	RELAPSE
20	RA	2	F	SUPRATENTORIAL	PRIMITIVE
21	SL	2	M	INFRATENTORIAL	RELAPSE
22	SJ	1	M	INFRATENTORIAL	PRIMITIVE
23	GF	4	F	INFRATENTORIAL	RELAPSE
24	VCA	1	F	INFRATENTORIAL	PRIMITIVE
25	ZFI	17	M	CAUDAL SPINAL CORD	METASTASIS

Abbreviations: ID = identification number; Pz = patient; M = male; F = female.

## Methods

Histologic diagnoses were made at the Department of Human Pathology and Oncology at the University of Florence and were performed according to the World Health Organization (WHO) criteria [16].

A series of six brain tissues obtained from six patients surgically treated because of non-neoplastic condition were used as control samples. According to institutional policies, the use of human tissue for study purposes was approved through a written informed consent.

From each fresh surgical specimen of anaplastic ependymomas, we selected a fragment macroscopically representative of the tumor. After doing so, we cut it in half: from one half several 5- $\mu$ m frozen sections stained with hematoxylin-eosin (H&E) were obtained to verify the adequacy of the specimen selected for real-time presence of pathologic tissue only or non-neoplastic brain parenchyma in cases that we used as control polymerase chain reaction (PCR) the other half was immersed in ribonucleic acid (RNA) later (QIAGEN, Valencia, CA), kept overnight at + 4°C, and finally stored at -80°C until analysis.

## Real-Time PCR

The tissue (about 5 mg) was defrosted and cut into small pieces. The samples were resuspended in 200  $\mu$ l of nucleic acid purification Lysis solution (Applied Biosystems, Foster City, CA) and homogenized using tissue Lyser (Qiagen, Milan, Italy) in microcentrifuge tubes. After proteinase K digestion (250  $\mu$ g/ml for 1 h at 50°C), RNA was isolated using a 6100 Nucleic Acid Prep Station (Applied Biosystems, Foster City, CA) and successively stored at -80°C.

The RNA concentration and purity was quantitated spectrophotometrically by measuring their absorbance at 260 nm and 280 nm. All RNA samples (200 ng) were reverse transcribed to cDNA using iScript Select cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's protocol. In the reverse transcription reaction, negative control was performed. Real-time quantitative PCR was performed on an ABI PRISM 7000 Sequence Detector System (Applied Biosystems, Foster City, CA). PCR products for NF2 were detected using gene-specific primers and probes labeled with reporter dye FAM (Assay on Demand, Applied Biosystems). gAPDH was used as endogenous control gene for normalization.

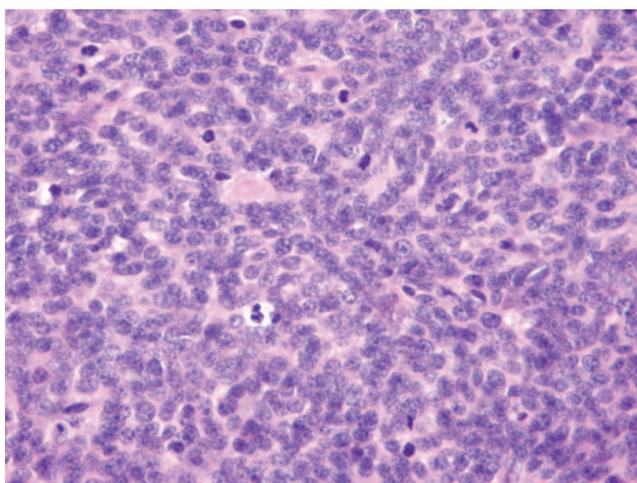
PCR reactions were carried out in 96-well plate with 20  $\mu$ l per well using 1x TaqMan Universal PCR Master Mix. After an incubation for 2 min at 50°C and 10 min at 95°C, the reaction continued for 50 cycles at 95°C for 15 sec and 60°C for 1 min.

## Results Analysis

At the end of the reaction, the results were evaluated using ABI 7000 PRISM software (Applied Biosystems) and the Ct values were exported to Microsoft Excel. The  $2^{-\Delta\Delta C_t}$  method described by Livak and Schmittgen [15] was used to calculate fold expression levels of all 25 ependymomas relative to the average value of the six non-neoplastic specimens, of 17 non-primitive (16 recurrences and 1 metastasis), anaplastic ependymomas relative to the average value of the eight primitive anaplastic ependymomas, and 15 supratentorial tumors relative to the average value of the eight 4th ventricular ependymomas.

## Statistical Analysis

The shift of the expression level of NF2 as estimated through relative RT-PCR was calculated according to the Kruskal-Wallis test. Data analysis was performed using the Primit statistical package, Version 3.03 (McGraw-Hill Inc, Milan, Italy). The AP value  $\leq 0.05$  was considered to be statistically significant.



**FIGURE 1** Anaplastic ependymoma (case 1) hypercellularity and numerous mitoses are appreciable. Hematoxylin-Eosin; original magnification  $\times 200$ .

## RESULTS

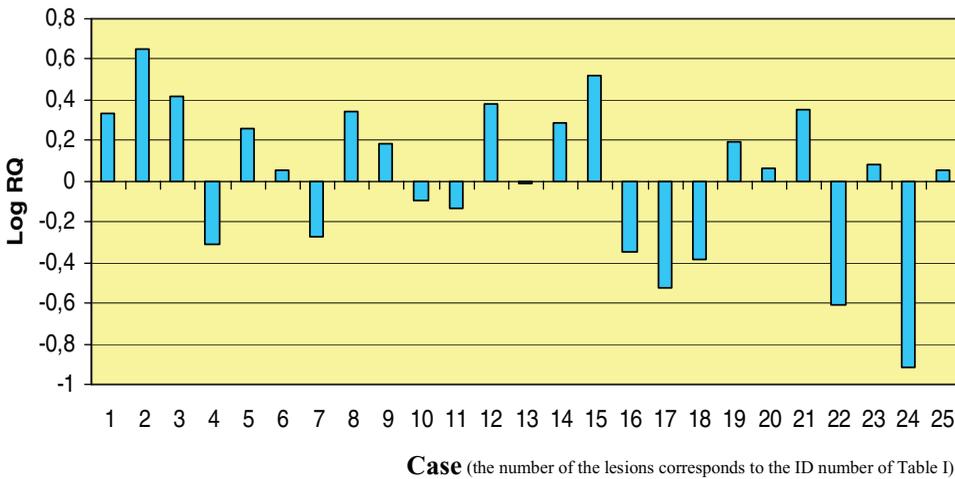
Fifteen (60%) anaplastic ependymomas were supratentorial, nine (36%) were infratentorial, and one (4%) was spinal. Eight (32%) lesions were primitive tumors, 16 (64%) were recurrences, and one (4%) was a caudal spinal cord metastasis. (Table 1)

We successfully extracted RNA from all samples. NF2 gene expression was present in all ependymomas and in all six non-neoplastic brain tissues. Fifteen (60%) tumor samples expressed the NF2 gene higher than non-neoplastic tissues (max 0,65 times higher) (Table 2; Figure 2). Eight (53%) supratentorial ependymomas expressed the NF2 gene lower than infratentorial ependymomas (max 0,56 times lower). (Table 3; Figure 3). Ten (59%) non-primitive lesions (nine recurrences and only one spinal metastasis) expressed the NF2 gene higher than primitive ependymomas (max 0,71 times higher) (Table 4; Figure 4).

Statistical analysis did not establish significant differences ( $P > 0.04$ ) between non-neoplastic or neoplastic specimens, supra or infratentorial, and primitive or non-primitive anaplastic ependymomas.

## DISCUSSION

Ependymomas are infrequent tumors of neuroectodermal origin classifying as World Health Organization (WHO) Grades I, II, and III. Ninety percent of ependymomas are intracranial and the majority of these arise in the posterior fossa.

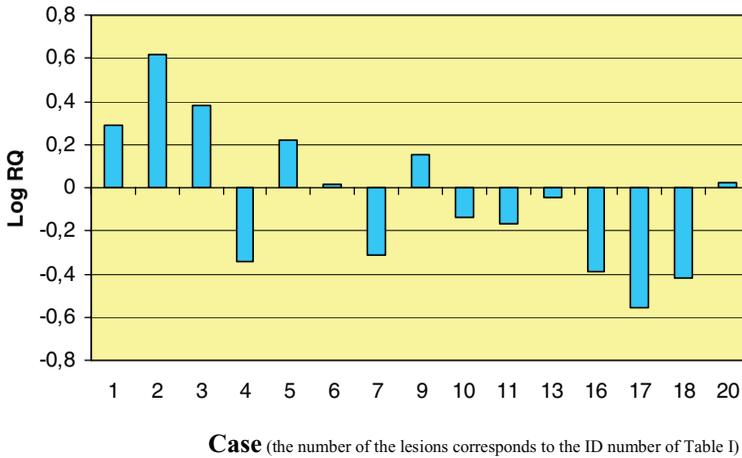


**FIGURE 2** RT-PCR merlin expression in 25 anaplastic ependymomas (reference value: mean merlin expression in 6 non neoplastic cerebral specimens). The number of the lesions corresponds to the ID number of Table 1.

**TABLE 2** RT-PCR: log RQ in 25 Anaplastic Ependymomas (Reference Value: Mean Merlin Expression in 6 Non Neoplastic Cerebral Specimens)

ID	Pz	log RQ
1	BM	0,329284
2		0,652891
3		0,420195
4	BA	-0,30619
5		0,254628
6		0,055045
7		-0,27518
8	CC	0,34584
9	FaF	0,186295
10		-0,09788
11		-0,13039
12	FrF	0,382867
13	LM	-0,00877
14	MG	0,286237
15		0,518933
16	MI	-0,34984
17		-0,52233
18	NT	-0,38235
19	RE	0,194121
20	RA	0,058959
21	SL	0,349152
22	SJ	-0,60963
23	GF	0,083342
24	VCA	-0,91548
25	ZFI	0,054744

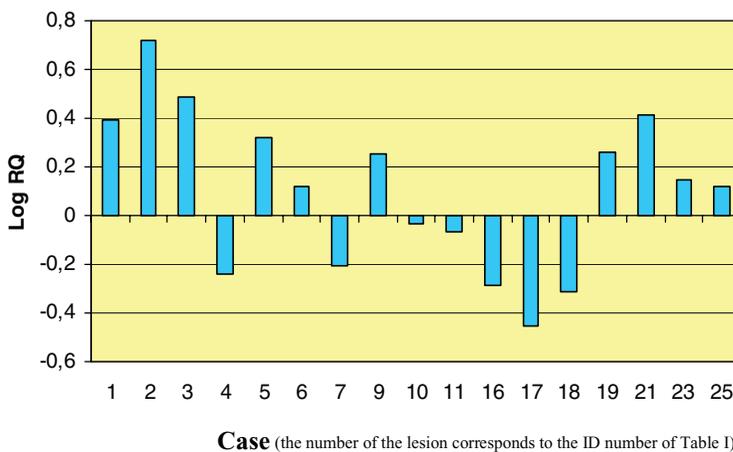
Abbreviations: ID = identification number; Pz = patient.



**FIGURE 3** RT-PCR merlin expression in 15 supratentorial anaplastic ependymomas (reference value: mean merlin expression in 9 infratentorial anaplastic ependymomas). The number of the lesions corresponds to the ID number of Table 1.

The single most important prognostic factor is the extent of tumor resection so that ependymoma is considered a “surgical disease.” In fact, the local relapses determine the majority of adverse outcomes. Nevertheless, it has been demonstrated that the histological tumor grade is also a predictor of the clinical behavior. Actually, WHO III anaplastic ependymomas show an unfavorable outcome [3].

Several molecular alterations have been reported in ependymomas, including aberrations involving chromosomes 1, 6, 7, 9, 10, 11, 12, 13, 16, 17, and the NF2 gene on chromosome 22q12 which encodes for merlin



**FIGURE 4** RT-PCR merlin expression in 17 non-primitive anaplastic ependymomas—16 recurrences; 1 caudal spinal cord metastasis (reference value: mean merlin expression in 8 primitive anaplastic ependymomas). The number of the lesions corresponds to the ID number of Table 1.

**TABLE 3** RT-PCR: log RQ in 15 supratentorial anaplastic ependymomas (reference value: mean merlin expression in 9 infratentorial anaplastic ependymomas)

ID	Pz	log RQ
1	BM	0,293263
2		0,616871
3		0,384174
4	BA	-0,34221
5		0,218608
6		0,019025
7	FaF	-0,3112
9		0,150274
10		-0,1339
11	LM	-0,16641
13		-0,04479
16		-0,38586
17	MI	-0,55835
18	NT	-0,41837
20	RA	0,022938

Abbreviations: ID = identification number; Pz = patient.

protein [21]. Mutations on the NF2 gene are considered the most frequent molecular alterations in ependymomas. Nevertheless, the analyses of mutations of the NF2 suppressor gene have yielded conflicting results. It has been postulated that NF2 mutations with consequent loss of merlin functions are related to spinal ependymomas only. Actually, in the opinion of some authors, spinal ependymomas could constitute a molecular variant

**TABLE 4** RT-PCR: log RQ in 17 non-primitive anaplastic ependymomas—16 recurrences; one caudal spinal cord metastasis (reference value: mean merlin expression in 8 primitive anaplastic ependymomas).

ID	Pz	log RQ
1	BM	0,394994
2		0,718602
3		0,485905
4	BA	-0,24048
5		0,320339
6		0,120756
7	FaF	-0,20947
9		0,252005
10		-0,03217
11	MI	-0,06468
16		-0,28413
17		-0,45662
18	NT	-0,31664
19	RE	0,259832
21	SL	0,414862
23	GF	0,149053
25	ZFI	0,120455

Abbreviations: ID = identification number; Pz = patient.

of ependymomas characterized by a high incidence of NF2 mutations. In 1994, in a group of 8 ependymomas (2 spinal and 6 intracranial; 4 WHO Grade II and 2 WHO Grade III) Rubio documented a single NF2 gene base deletion in a WHO II spinal ependymoma which also had lost the wild-type allele [20]. In 1996, Birch stated that 5 of 7 spinal ependymomas that he studied harbored mutations on the NF2 gene resulting in a truncation of the predicted protein product [2]. In 2002, Alonso stated that 1 anaplastic WHO III spinal ependymoma of her series of 7 ependimomas (2 spinal and 5 intracranial; 4 WHO Grade II and 3 WHO Grade III) showed mutation of the NF2 gene [1].

In our study, we compared the merlin expression in anaplastic intracranial ependymomas and in the non-neoplastic brain tissue. The observation that no significant differences in the merlin expression were found between ependymomas of our series (24 intracranial and 1 caudal cord metastasis) and non-neoplastic cerebral parenchyma might indirectly support the hypothesis that NF2 alterations are unusual in intracranial ependymomas (both infratentorial and supratentorial ependymomas as also evaluated by comparing their expression).

Furthermore, among ependymomas with NF2 alterations reported in the literature, there were a number of anaplastic ependymomas. This observation stimulates us to further exclude the possibility of a grade related phenomenon. In this regard, we confirm that modifications in the NF2 expression did not characterize anaplastic ependymomas.

On the other hand, we can also suppose that the alterations in NF2 are not more frequent in a relapsed lesion than in primary tumors. Indeed, we did not observe a significant difference in primitive and non-primitive ependymomas.

In conclusion, although the method of research that we used (evaluation of the merlin expression through RT-PCR) did not assess NF2 gene aberrations, our results may indirectly provide evidence that NF2 gene mutations do not play an important role in the aetiology and progression of intracranial anaplastic ependymomas.

## DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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