Prediction of Prognosis in Medulloblastoma using Immunohistochemical Analysis and Tissue Microarray.

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Prediction of Prognosis in Medulloblastoma using Immunohistochemical Analysis and Tissue Microarray.

Directed by Professor Dong-Seok Kim

The Master's Thesis submitted to the Department of Medical Science, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Master of Medical Science

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June 2006

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June 2002

ACKNOWLEDGEMENTS

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그리고 석사과정 내내 옆에서 땡땡이 치고 놀자던 나의 사랑스러운 변덕쟁이 반쪽양, 미래의 판검사 바쁜쟁이, 보고 싶은 Sydney Uni colleagues, 나의 Sydney 빽 (無)경우오빠, 14년 지기 엉뚱소녀, 애교만점 하치군, 확실한 내 편 스뻔지밥, 손녀 끼니 걱정에 애 닳던 할머니, 깍쟁이 야시이모와 딸들, 나의 압구정family, 씩씩한 현서 엄마, 또 멀리 있지만 생각만으로도 늘 웃음 주며 행복 주는 사랑스러운 My brother! 철우야 보고 싶다!
마지막으로 이 세상에 존재할 수 있고, 이 자리에 서 있을 수 있도록 만들어준 엄마, 아빠 정말 존경하고 사랑합니다.
나 석사 끝냈어요!
엄마, 아빠에게도 제가 자랑스러웠으면 좋겠습니다. 하느님께서 보시기에도 제가 예뻐 보였으면 좋겠습니다.

2006년 6월
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ABSTRACT

Prediction of prognosis in medulloblastoma using immunohistochemical analysis and tissue microarray

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Medulloblastoma (MB) is the most common malignant neuroepithelial tumor found in the childhood. During last 20 years, novel therapeutic modalities for MB have been developed in an application of craniospinal irradiation and adjuvant chemotherapy that have significantly improved MB outcomes with 5-year survival rate up to 70-80%. However the most important treatment of chemotherapy for MB is at high risk for development of crude side effects. Therefore identifying the suitable therapeutic approaches in an attempt to improve cure rates and minimize the side effects is utmost important. Therefore to define prognosis of patients is important to determine the clinical factors and also to identify the biological markers that could be useful in

prediction of MB prognosis and further clinical subdivision of patients and several reports describe certain efforts to identify the prognostic significance of various patterns of MB pathology and immunohistochemistry but received data appear to be controversial. Hence we attempted to convincingly demonstrate on 58 MB patients with age above 3 and performed one year follow up after the operation which they undergone maximal surgical resection, craniospinal radiation therapy and chemotherapy to analyze the immunohistochemical features to cellular differentiation, the proliferative index (PI), the apoptotic index (AI), and oncogenesis of TrkC and c-erbB-3 to determine their prognostic utility in this tumor category. In our result that there was no significant correlation between the prognosis and the degree of cell differentiation but the positive correlation was noted between PI and AI in a tumor mass and the number of cases with PI > 20% was significantly greater in the group of tumors in patients with recurrent MB. A close association between PI as a continuous variable and the progression-free and overall survival was found that PI is a single significant prognostic factor for MB survival.

Key Words: Medulloblastoma, Proliferative index, Prognosis, TrkC

Prediction of prognosis in medulloblastoma using immunohistochemical analysis and tissue microarray

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I. INTRODUCTION

Medulloblastoma is a malignant primitive neuroectodermal tumor of the cerebellum, which preferentially occurs in children (accounting for 20-25% of pediatric brain tumors), and has a tendency to metastasize via cerebrospinal fluid (CSF) pathways[1, 2, 3]. According to recent data (Fig 1), an application of craniopinal irradiation and adjuvant chemotherapy have significantly improved medulloblastoma outcomes with 5-year survival rate up to 60-70%[4, 6] and of course, more aggressive treatment modalities are still required to

increase the survival rates of medulloblastoma children. Nevertheless, we should consider the treatment complications in the long-term survivals such as impairment of neurocognitive function, endocrine dysfunction, and secondary tumors. Therefore, a proper identification of prognostic and predictive factors on which to stratify patients in risk groups is, therefore, of utmost importance for planning adequate treatment and trying to avoid the dysfunctions[20, 21].

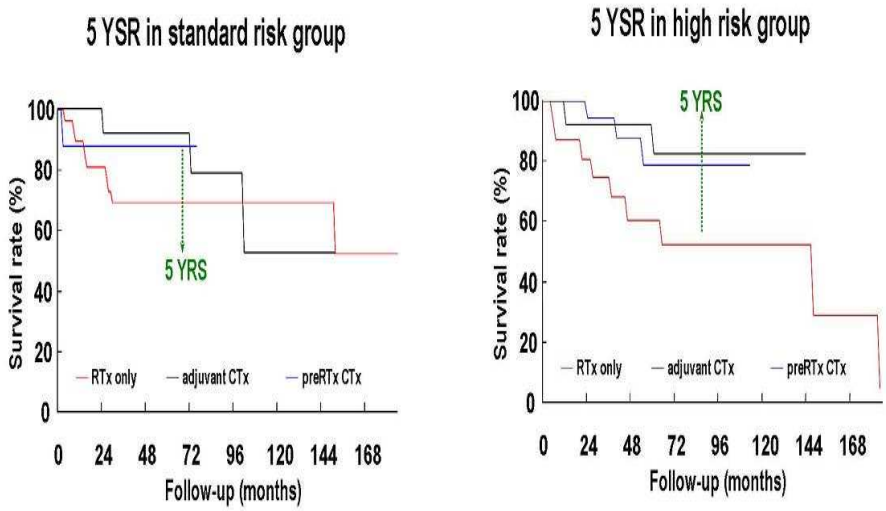


Figure 1. The 5-year survival rate of medulloblastoma in standard and high risk group. Recently, an application of craniopinal irradiation and adjuvant chemotherapy has significantly improved medulloblastoma outcomes with 5-year survival rate up to 60-70%. (In our data, diagnosed children from 1990 to 2004. Yonsei University Medical Center, Korea.)

Our data showed there is no difference in the survival rates according to the

treatment modalities in standard risk group. However, in high risk group, pre-irradiation or adjuvant chemotherapy is sure to increase the survival rate and it indicate that intensity of treatment should be different depending on the predicted prognosis to control the tumors with minimal complications. The clinical prognosis factors of pediatric medulloblastoma have been well studied over the past 20 years and currently, few clinical factors have been identified as rough indicators of unfavorable medulloblastoma outcome[3]. Factors that correlate with outcome include age at diagnosis, the completeness of surgical resection, presence or absence of cerebrospinal fluid (CSF) seeding at diagnosis and extraneural metastasis. The presence of brainstem involvement has been correlated to poor prognosis in past studies, but in more recent studies has not been associated with disease progression.

The patients are now commonly grouped according to their clinical presentation into standard (older than the age of 3 years with M0 and residual post-operational tumor smaller than 1.5㎠) and high-risk (younger than the age of 3years with M+ and residual post-operational tumor bigger than 1.5㎠) groups[7, 30], which helps guide therapeutic decision. For the high-risk patients may allow more aggressive therapeutic approaches in an attempt to improve cure rates and conversely, standard-risk patients might allow less aggressive therapies in attempt to decrease morbidity[17, 19]. However, we have experienced

lots of exceptional cases showing unpredicted prognosis, so that numerous attempts have been made to identify the biological markers that could be useful in prediction of medulloblastoma prognosis and further clinical subdivision of patients.

Immunohistological techniques are potential powerful tools for risk stratification which are used in several tumor factors in addition to clinical factors which it includes differentiation, large cell, anaplastic variant, proliferation index, and apoptosis of tumor cells that were recently recognized to associate with advanced stage and poor prognosis[2]. Present clinical stratification has proven useful as a broad guide for predicting prognosis and consequently, efforts are being made to identify patients who can be cured with less intensive therapy and also to develop more effective treatments for children with resistant disease by the combination of prognostic significance, and its association with histological type and clinical parameters.

We suggested that these tumor factors may be useful parameters in classifying the prognosis of medulloblastoma and planed this study to identify their biological significance. Traditionally, comparing multiple markers on multiple tumors, although possible, was logistically challenging. However, the advent of the tissue microarray, a recently developed technology allowing hundreds of tissue sections from different tumors to be arrayed on a single glass slide,

has made this task far less forbidding. Not only does this facilitate rapid evaluation of large-scale outcome studies, it also allows comparison of histologic features, DNA sequence, and transcript expression on contiguous sections of the same tumor[30]. Furthermore, multiple positive and negative controls included on each slide serve to standardize the immunohistochemical staining.

In present study, a medulloblastoma tissue microarray is used to assess the prognostic significance of multiple immunohistochemical markers in 58 medulloblastoma tumors correlated with patient outcome and analyses are carried out to assess the significance of these markers alone and in combination with previously established by which both clinical and biological information can be combined to estimate patient survival.

II. MATERIALS AND METHODS

1. Patients and Tumor Specimens

To maximally eliminate the clinical factors in our study, we excluded all cases with less than age of 3 years and any surgical morbidity and mortality. All patients completed the same treatment schedule as surgery, radiation, and adjuvant chemotherapy. After with approval of institutional review board and obtaining signed informed consent, we finally obtained 58 samples of paraffin-blocked medulloblastoma which it operated at Yonsei University Medical Center, Korea from diagnosed children between 1990 and 2004. The samples were obtained at the time of primary surgical resection, stored at -80°C for study references and some of samples were obtained as via primary culture of brain tissues. All samples were de-identified by assignment of a study number and laboratory staff and the neuropathologist (C.F.) were masked to all patient clinical details so that all the clinical features of medulloblastoma children have been recorded in each case. The classification of tumor by subtype (i.e. classic, desmoplastic/nodular, and large cell/anaplastic) was performed using World Health organization guidelines[11, 22] and formalin-fixed paraffin-embedded samples of each tumor were also collected and subjected to central histopathology review by a single

neuropathologist (C.F). The clinical details of each patient in the study cohort were reviewed by a single neuro-oncologist (A.G.) who was masked to all histopathologic and molecular analyses. 47 cases with disease free survival at the last follow-up were defined as good prognosis, and 11 cases with any tumor recurrence or disease related death were defined as poor prognosis.

2. Construction of the Medulloblastoma Tissue Microarray

The formalin fixed and paraffin embedded tissues were used for tissue microarray and for each patients, all pathological blocks and corresponding slides were obtained then reviewed by neuropathology for diagnostic accuracy and tissue adequacy. Representative tumor areas were identified, and between two and three cores were obtained for each tumor, giving a sampling accuracy of at least 95%[30, 31, 32]. The blocks (Fig 2) with different samples of each medulloblastoma prepared in 5㎛ sections and mounted on positively charged microscope slides (Fig 3).

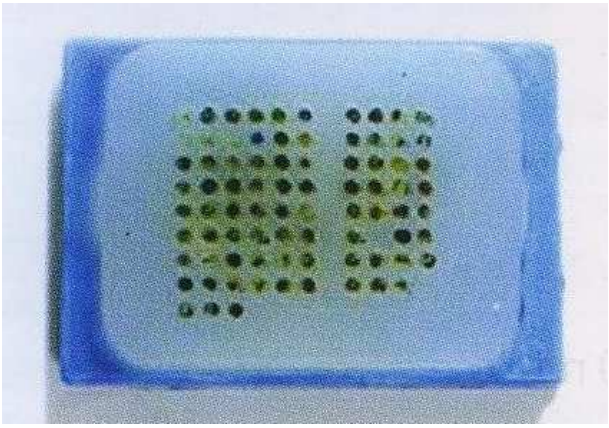


Fig 2. The tissue microarray block. It contains two and three cores of each tumor samples

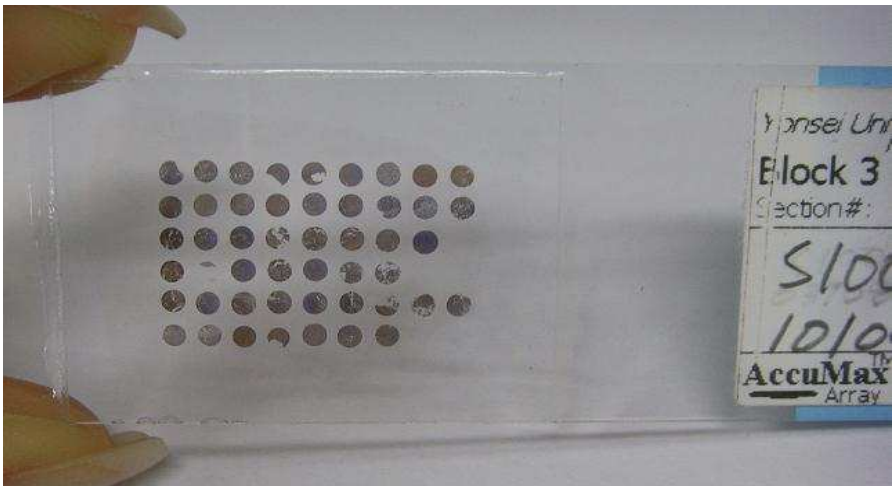


Fig 3. The tissue microarray slide. 5㎛ sections were cut from the tissue microarray and mounted on slides then it stained.

3. Histology and Immunohistochemical Analysis

All the samples were reviewed for focused on nuclear size and anaplasia via routinely stained of hematoxylin and eosin (H&E) and in all cases, immunohistochemical analysis were performed on representative 5㎛ thick tissue sections. Tissue sections were then baked for 1hr at 60℃, dewaxed in xylene, rehydrated in graded alcohol, and hydrated with distilled water through decreasing concentration of alcohol. The blockage of the endogenous peroxidase activity was done by 3% H_2O_2 in water and antigen retrieval was carried out by pressure-cooking in citrate buffer, pH 6.0. The appropriate antibodies and working dilutions are listed in table 1. After incubation (1hr at room temperature then overnight at 4℃) with primary antibodies, sections were washed and treated with biotinylated link antibody for 20min. Antigen visualization was achieved by applying a streptavidin-HRP complex (LSAB kit, Dako, Denmark) for 20min followed by 3, 3'-diaminobenzidine chromogen (DAB, Dako, Denmark) in substrate buffer (imidazole-HCl buffer pH 7.5 with H_2O_2 and anti-microbial agent). Sections were counterstained with haematoxylin. The specificity of the antibodies used was checked with positive and negative control sections. Sections treated without the primary antibodies served as negative controls. For positive controls, neural cell adhesion molecule (NCAM), Neurofilament (NF), Glial fibrillary acidic

protein (GFAP), caspase-3; brain, Vimentin, Ki67; tonsil, epithelial membrane antigen (EMA), erB-3; breast carcinoma, nerve growth factor receptor (NGFR), S100; malignant melanoma, Synaptophysin; pancreas tissue, Bcl-2; follicular hyperplasia and TrkC; spinal ganglion was processed in the same way as the medulloblastoma sides. Sections were reviewed independently, which they were analyzed at high and low power. All scoring was done in a blinded fashion with no knowledge of patient outcome and the scoring for immunoreactivity of each antibody reflects the percentage of staining within representative areas of each tumor and followed scoring system that – indicates negative, 1+ indicates 10%, 2+ indicates 10-50% and 3+ indicates >50%[18].

Table 1. Summary of antibodies used in this study

Antibodies	Clone/Dilution	Source
NGFR	NGFR 5/1:50	DAKO, Carpinteria, CA, USA
Neurofilament	DA2, FNPT, Rmdo 20.11/1:100	Zymed, San Francisco, CA, USA
Synaptophysin	Polyclonal/1:200	Shandon, Pittsburgh, PA, USA
S100	Polyclonal/1:400	DAKO, Carpinteria, CA, USA
p53	DO-7/1:100	Neomarkers, Fremont, CA, USA
GFAP	6F2/1:100	DAKO, Carpinteria, CA, USA
Bcl-2	124/1:50	DAKO, Carpinteria, CA, USA
Ki67	MM1/1:100	Novocastra, Newcastle upon Tyne, UK
c-erbB-3	RTJ1(2E11)/1:100	Chemicon, Temecula, CA, USA
Caspase 3	E-8/1:100	Santa Cruz, CA, USA
EMA	E29/1:40	DAKO, Carpinteria, CA, USA
TrkC	Polyclonal/1:400	Santa Cruz, CA, USA
Vimentin	V9/1:100	DAKO, Carpinteria, CA, USA

NGFR, nerve growth factor receptor; GFAP, glial fibrillary acidic protein; EMA, epithelial membrane antigen.

4. **Primary Culture in Medulloblastoma**

The sample are taken biopsy, at the time of surgery and set up as a sterile monolayer culture. They are collected aseptically in DMEM/F12 (Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12) and separated into two parts; tissue culture and cell culture. For tissue culture, the sample was fixed in 10% Paraformaldehyde for 24 hours then made into paraffin embedded blocks that is used for immunohistochemistry.

For cell culture, mince the sample with scalpels into small cubes approximately $0.5mm^3$ and transfer the minced sample pieces to the test tubes (15ml) and wash sample three times in Hank's BSS with 3g/l BSA (HBSS). Allow the sample to settle to the bottom of the tubes between each washing and then incubated with 0.025% trypsin (in HBSS) for 30 minutes at 37℃ with gentle shaking. Digestion was stopped by growth medium then the cells were passed through 70㎛ nylon cell strainer (Falcon) sequentially. Cells were resuspended in DMEM with 4mM L-glutamine, 25mM glucose, 10% FBS(all from Gibco), plated in T75㎠ plastic tissue culture flask at a concentration of 4 or 8×10^6 cells per flask. Cultures were kept in a 5% CO_2/95% air incubator at 37℃ for 10days with regular changes of culture medium.

5. Statistical Analysis

The chi-square test was performed to determine whether the relationships were statistically significant. Associations among clinical features (gender, age at diagnosis, metastatic disease stage, surgery extent, amount of residual tumor and clinical risk group), histopathology, and molecular characteristics were investigated using Fisher's exact test or χ^2 test for multivariate analysis with discrete parameters.

Progression-free survival (PFS) was calculated from the day of surgical interference till the last event and overall survival (OAS) was calculated from the first day of treatment to the last follow-up date or death. For each biological and clinical marker, the association with survival rates was characterized using Kaplan-Meier method. For Ki-67 labeling index result were expressed by mean $\pm$ SD and the mean level were compared using the Student’s unpaired t-test. A P-value of <0.05 was considered statistically significant

III. RESULTS

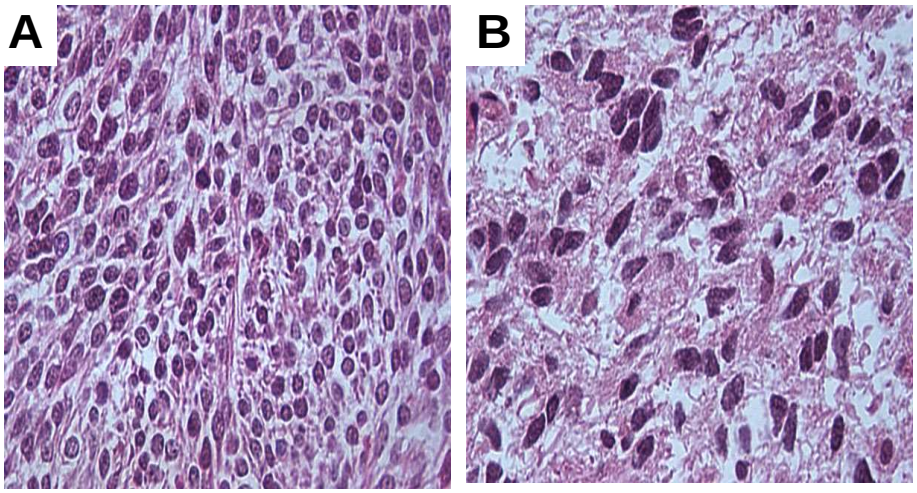
1. Clinical features of patients

In total, 58 patients were included in the study (42males and 16 females). The age at the present ranged from 3years to 34 years, with a mean age at presentation of 8.4 years (median, 7 years). Extent of tumor at the time of diagnosis, as designated by T stage, was T1 in two patients, T2 in four patients, T3 in forty-three patients, and T4 in nine patients. Twenty-eight patients were staged as M0, twelve were of M1 stage, fifteen were of M3 stage, and three were of M unknown stage. All patients in our sample had no major surgical morbidity or mortality and performed one year follow up after the operation which they undergone maximal surgical resection, craniospinal radiation therapy and chemotherapy. We defined prognosis in two; good (no recurrence and alive) and poor (recurrence or dead) after the treatment. As a result of prognosis in 58 medulloblastoma patients that 47 cases were good and 11 cases were poor. Hence we could not find any significant difference in prognosis of Medulloblastoma according to the clinical prognostic factors such as tumor size, resection extend, and presence of metastasis.

2. Histopathological features and prognosis

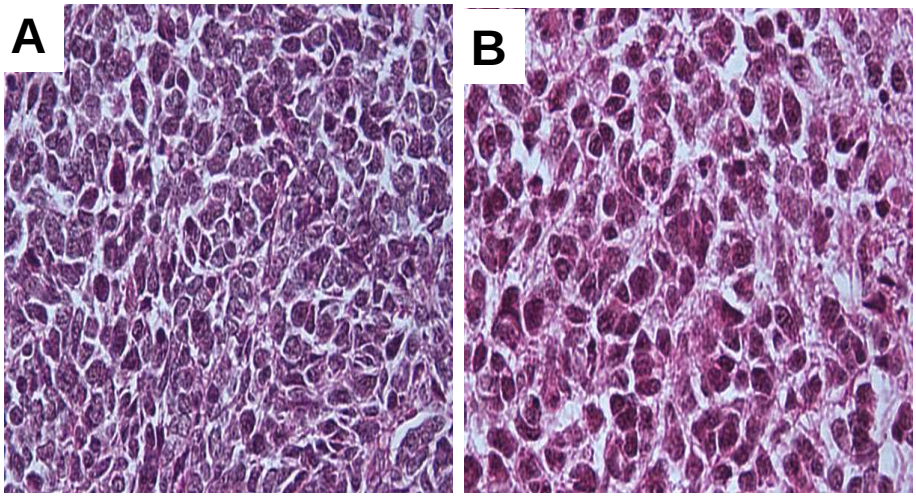
The review of H&E stain is focused on nuclear size and anaplasia. For nuclear size, we counted more than ten spots from each stained tissues and then averaged counted values. Among these samples that forty-nine cases were classified as classic and nine cases were large cell medulloblastoma (Fig 4). The average value of nuclear size for large cell were 15.4㎛ and classic type was characterized by a diffuse growth of tumor cells without formation of nodules and tumor cells with large, pleomorphic nuclei were classified as large cell medulloblastoma. The nuclear size values over the prognosis values of these series did not predict anything and no differences were observed.

Forty-seven cases were anapalsia and eleven cases were non-anaplasia in medulloblastoma (Fig. 5). We counted anaplastic cells on 10 high power fields and the anaplastic subtype, the degree of anaplasia was graded as none (counted cells less than 4), slight (counted cells between 4 to 10), or severe (counted cells more than 10) based on increasing degrees of nuclear size, numerous mitoses and apoptoses, and large cell with round and prominent nuclei or angular, crowded nuclei in large cells that sometimes wrapped around one another[14]. However, anaplasia did not influence the prognosis and further that the classic and large cell medulloblastoma had almost same prognosis.



Classification	**Nuclear size**		**Prognosis**	
	Range	Mean	Good	Poor
Classic	3.5-11.6 ㎛	7.65 ㎛	39	10 (20%)
Large	13.3-18.9 ㎛	15.39 ㎛	8	1 (11%)

Fig 4. Classification of medulloblastomas according to nuclear size. (H&E stain with ×400). A) The Classical medulloblastomas are densely packed small cells with round-to-oval or carrot-shaped highly hyper chromatic nuclei surrounded by scanty cytoplasm. B) The large cell medulloblastomas with extremely large and hyper chromatic nuclei wrapped by abundant cytoplasm. Nuclear size had no significant in prognosis of medulloblastoma (P=0.81).

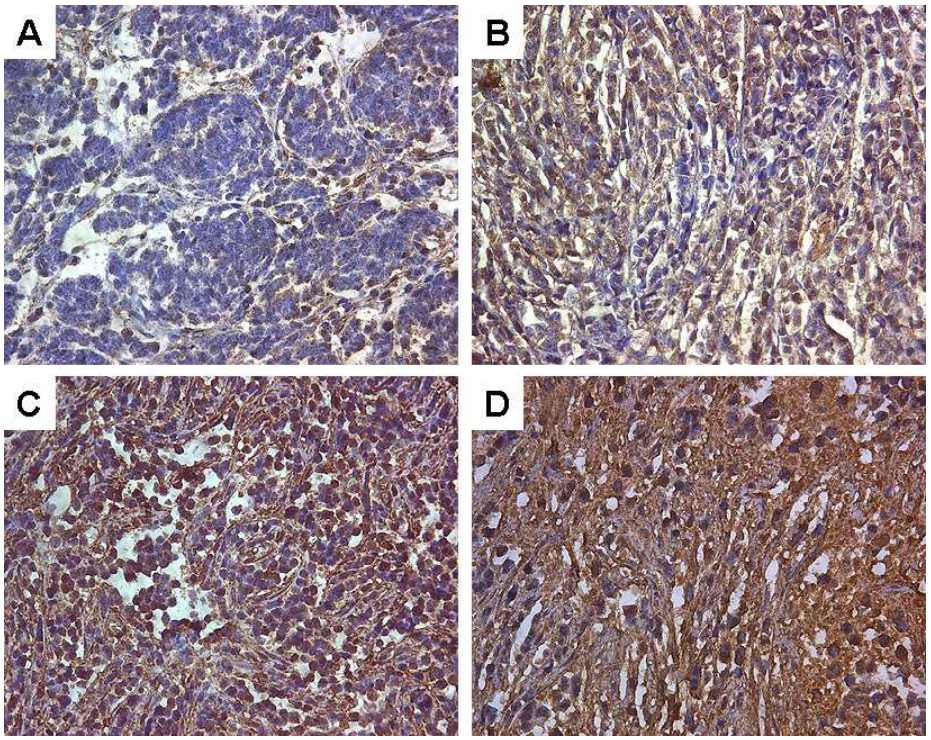


Anaplasia			Prognosis	
Grade	Range	Mean	Good	Poor
None (<4/10 HFP)	1-15	5.7	10	1 (9%)
Slight (4-10/10HFP)	1-29	12.5	20	8 (28.6%)
Severe (>10/10HFP)	4-47	26.5	17	2 (11%)

Fig 5. The H&E staining of medulloblastoma cells relationship to anaplasia and prognosis (x400). A) Non-anaplastic medulloblastomas contain nuclei smaller than those found in anaplastic lesions and often with angular molding. B) Nuclear enlargement was the principal feature of anaplastic medulloblastomas and wrapping of cells were prominent. In prognosis of medulloblastoma, the anaplasia had no significance. (p=0.73)

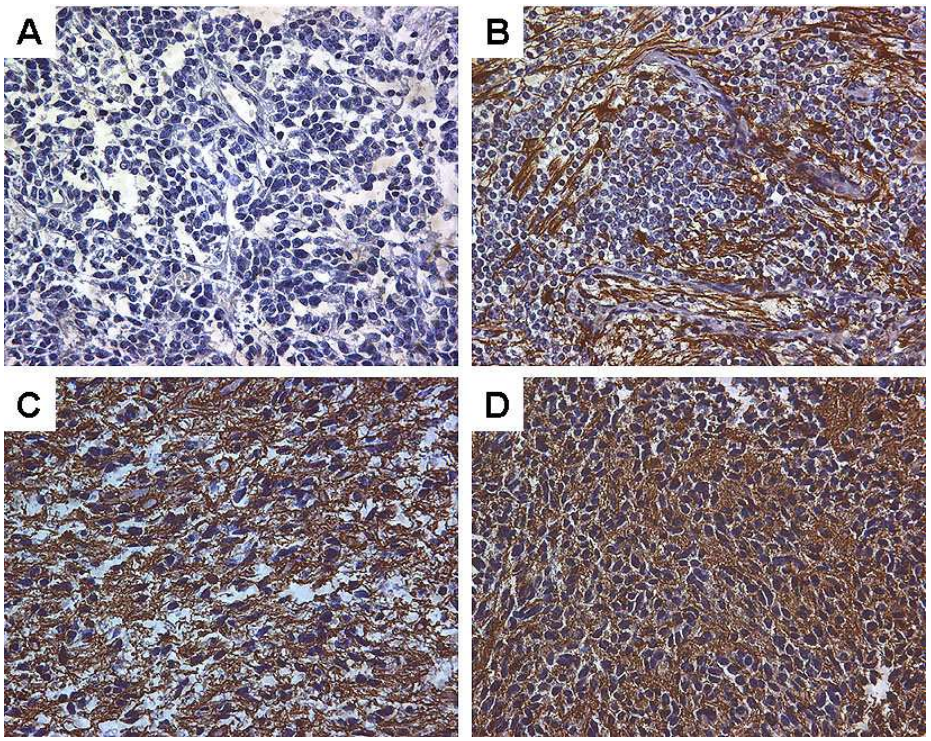
3. Comparison of cell differentiation and prognosis

Several antibodies were used to define the differentiation of 58 medulloblastoma patients into such as neuronal, mesenchymal, glial, epithelial, and mixed. The defined differentiations of patients are summarized in Table 2 and the percentage of medulloblastomas positive for each of the immunostains was as follows: NF, 67% (39 of 58); NGFR5, 43% (25 of 58); Vimentin, 84% (49 of 58); EMA, 90% (52 of 58); Synaptophysin, 84% (49 of 58); GFAP, 91% (53 of 58); and S100, 97% (56 of 58). For the scoring of all of each antibody reflects the approximate area (as a percentage) of each tumor sample that contained positively labeled tumor cells according to the following scoring system:-, negative; 1+, 10%; 2+, 10-50%; 3+, >50%[18]. The most of the cases were classified as mixed differentiation thus we experimented on each type of differentiations to find a relationship to each of their prognosis. The S100 was present in almost all patients and it analyzed to be a mixed differentiation which it correlated with features in astrocytic and neuronal (Fig 6). The glial fibrillary acidic protein (GFAP) exhibits the fibrillar pattern within nodules, expressing glial differentiation in two patients which it disclosed cell similarly in distribution to those seen with S100 (Fig 7)



Immunoreactivity	Good prognosis	Poor prognosis
Negative (Fig A)	**2 (100%)**	**0 (0%)**
Positive	**45 (80.4%)**	**11 (19.6%)**
1+ (Fig B)	1	2
2+ (Fig C)	15	2
3+ (Fig D)	29	7

Fig 6. Immunoreactivity of medulloblastoma cells to S100. It is confined within the nodules that strongly suggestive of the glial cell differentiation. The scoring was negative, 1+, 2+, 3+ from (A) to (D) respectively (x 400) and P=0.77.



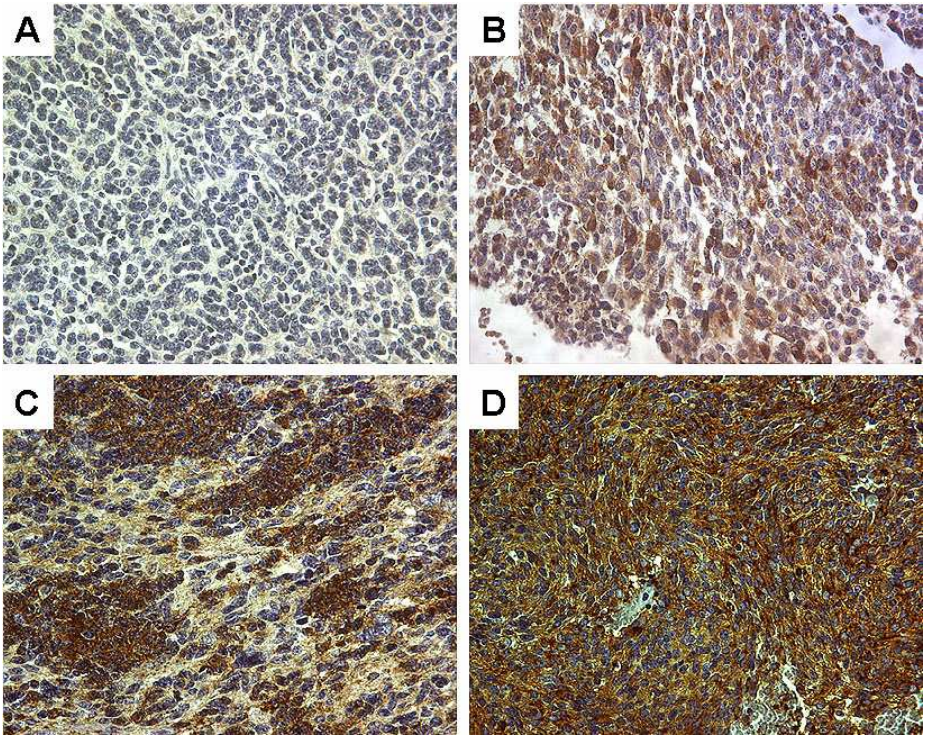
Immunoreactivity	Good prognosis	Poor prognosis
Negative (Fig A)	**4 (80%)**	**1 (20%)**
Positive	**43 (81.1%)**	**10 (18.9%)**
1+ (Fig B)	21	7
2+ (Fig C)	12	2
3+ (Fig D)	10	1

Fig 7. Immunoreactivity of medulloblastoma cells to GFAP for glial differentiation. It is exhibiting a simplified fibrillar pattern within the nodule. The scoring was negative, 1+, 2+, 3+ from (A) to (D) respectively (x 400) and p=0.69.

Table 2. Differentiation of 58 medulloblastoma patients and prognosis

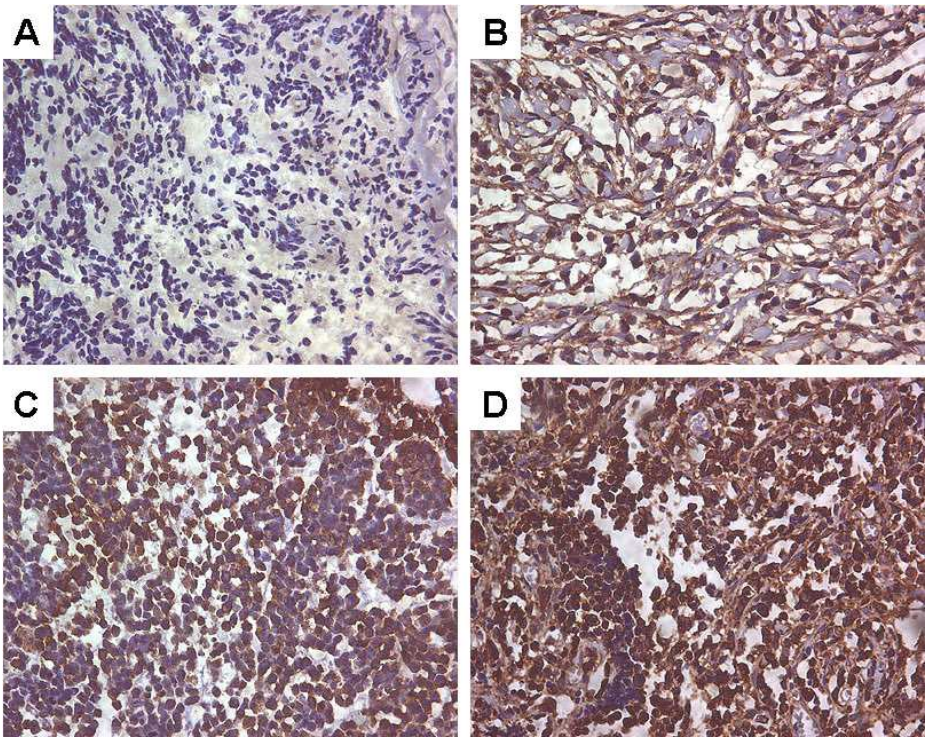
Differentiation	No. of patients	Poor prognosis
Neuronal	5 (9%)	3 (27%)
Mesenchymal	9 (16%)	3 (27%)
Glial	2 (3%)	0
Epithelial	2 (3%)	0
Mixed	40 (69%)	5 (45%)

All medulloblastomas exhibited neuronal differentiation of Synaptophysin reactivity at least focally and it was strongest within the cytoplasm of cells within nodule (Fig 8). Similarly in Neurofilament (NF) and nerve growth factor receptor (NGFR5) exhibited similar neuronal differentiation in cytoplasmic of tumor cells with fine filamentous pattern within nodules of medulloblastoma (Fig 9). However immunoreactivity of NGFR5 was weakly observed in this assay (Fig 10) and five patients were defined to neuronal differentiation and among these numbers three patients (27%) were defined as poor prognosis.



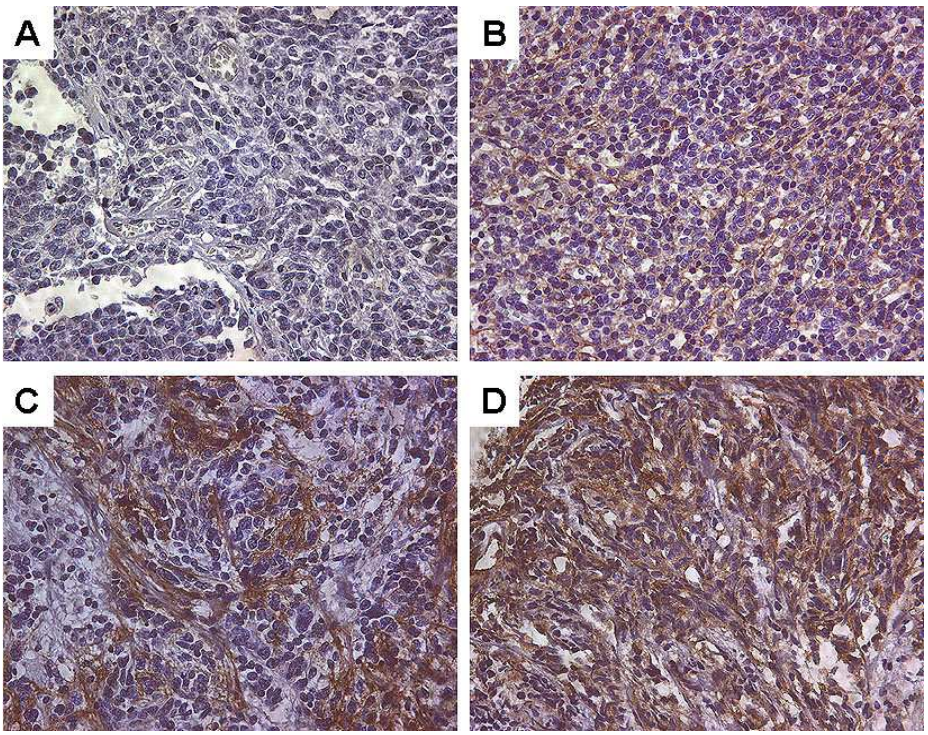
Immunoreactivity	Good prognosis	Poor prognosis
Negative (Fig A)	**6 (66.7%)**	**3 (33.3%)**
Positive	**41 (83.7%)**	**8 (16.3%)**
1+ (Fig B)	15	2
2+ (Fig C)	17	5
3+ (Fig D)	9	1

Fig 8. Immunoreactivity of medulloblastoma cells to synaptophysin for neuronal differentiation. It is noted to increased intensity within the cytoplasm of cells within nodule. The scoring was negative, 1+, 2+, 3+ from (A) to (D) respectively (x 400) and p=0.48.



Immunoreactivity	Good prognosis	Poor prognosis
Negative (Fig A)	**17 (89.5%)**	**2 (10.5%)**
Positive	**30 (76.9%)**	**9 (23.1%)**
1+ (Fig B)	18	6
2+ (Fig C)	5	0
3+ (Fig D)	7	3

Fig 9. Immunoreactivity of medulloblastoma cells to NF for neuronal differentiation. It stained with fine filamentous pattern within the nodules. The scoring was negative, 1+, 2+, 3+ from (A) to (D) respectively (x 400) and p=0.51.

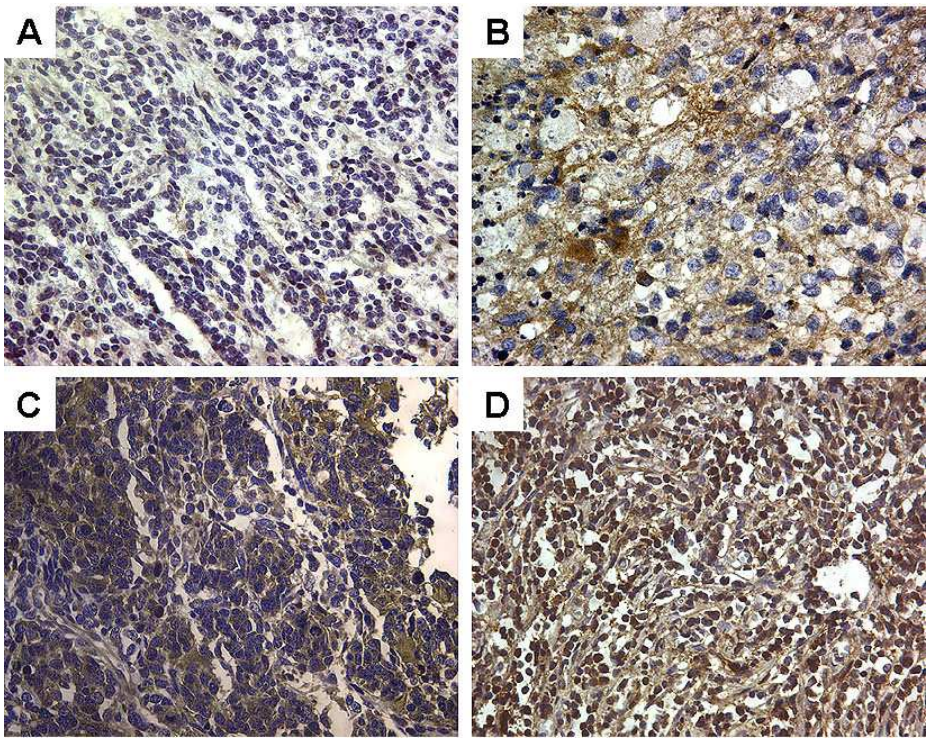


Immunoreactivity	Good prognosis	Poor prognosis
Negative (Fig A)	**28 (84.8%)**	**5 (15.2%)**
Positive	**19 (76%)**	**6 (24%)**
1+ (Fig B)	13	3
2+ (Fig C)	5	2
3+ (Fig D)	1	1

Fig 10. Immunoreactivity of medulloblastoma cells to NGFR5 for neuronal differentiation. It showed cytoplasmic immunoreactivity in small and larger cells whereas the nerve fibers are stained positive. The scoring was negative, 1+, 2+, 3+ from (A) to (D) respectively (x 400) and p=0.57.

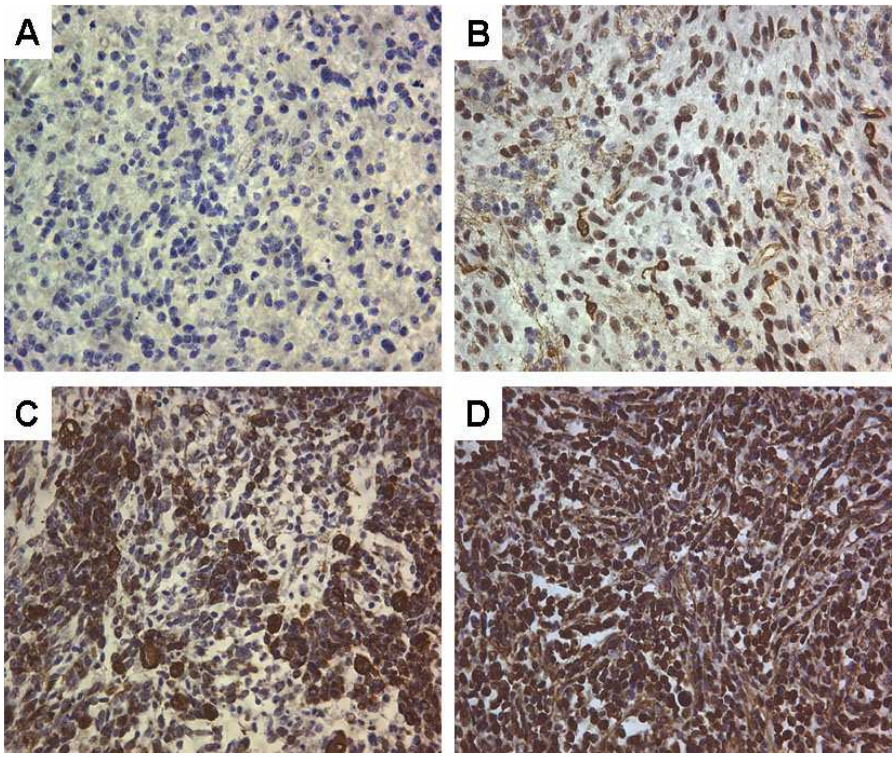
The epithelial membrane antigen (EMA) revealed epithelial differentiation which it stained fibrous around glia and it seems that the backgrounds of cells are stained and there was only two patients were defined in this array (Fig 11). In immunoreactivity that focal Vimentin positivity was noted in many cases which it revealed mesenchymal differentiation (Fig 12) and it exhibited the similar expression of GFAP that stained within nodules.

Through out these results the staining of each differentiation of tumor mass and the values of prognosis are almost same among these different differentiations in different patients. Therefore consequently it is clear that the differentiation does not reveal any signification of prognosis toward medulloblastoma patients.



Immunoreactivity	Good prognosis	Poor prognosis
Negative (Fig A)	**3 (50%)**	**3 (50%)**
Positive	**44 (84.6%)**	**8 (15.4%)**
1+ (Fig B)	9	1
2+ (Fig C)	16	3
3+ (Fig D)	19	4

Fig 11. Immunoreactivity of medulloblastoma cells to EMA for epithelial differentiation. It seems as that the backgrounds of cells have been stained positive. The scoring was negative, 1+, 2+, 3+ from (A) to (D) respectively (x 400) and p=0.73.

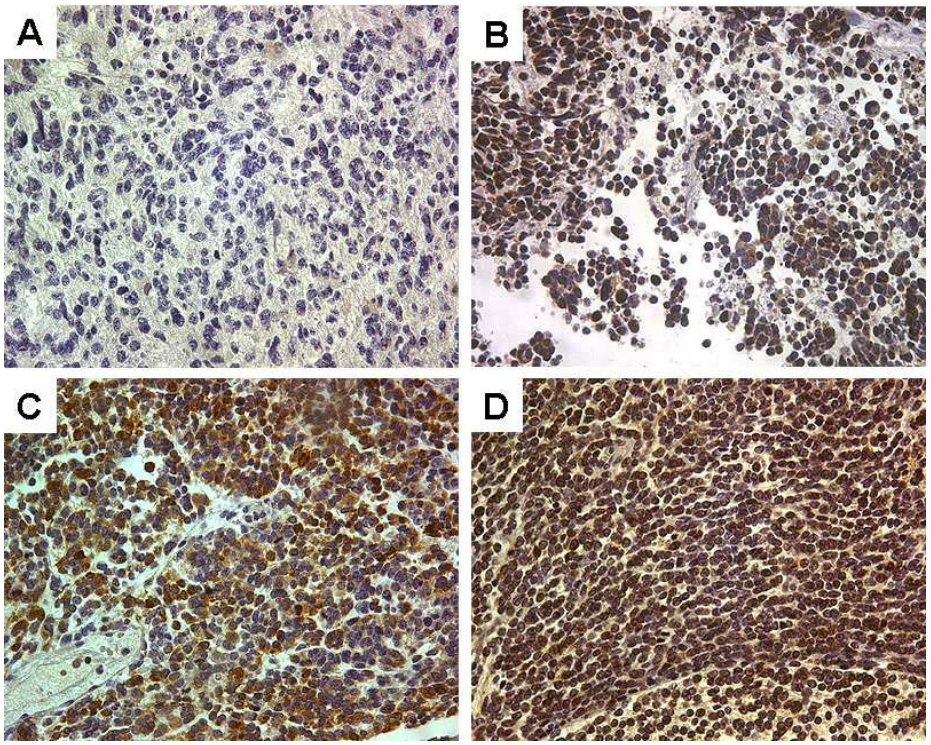


Immunoreactivity	Good prognosis	Poor prognosis
Negative (Fig A)	**7 (77.8%)**	**2 (22.2%)**
Positive	**40 (81.6%)**	**9 (18.4%)**
1+ (Fig B)	0	2
2+ (Fig C)	15	0
3+ (Fig D)	25	7

Fig 12. Immunoreactivity of medulloblastoma cells to vimentin for mesenchymal differentiation. The positivity was noted in almost all cases. The scoring was negative, 1+, 2+, 3+ from (A) to (D) respectively (x 400) and p=0.68.

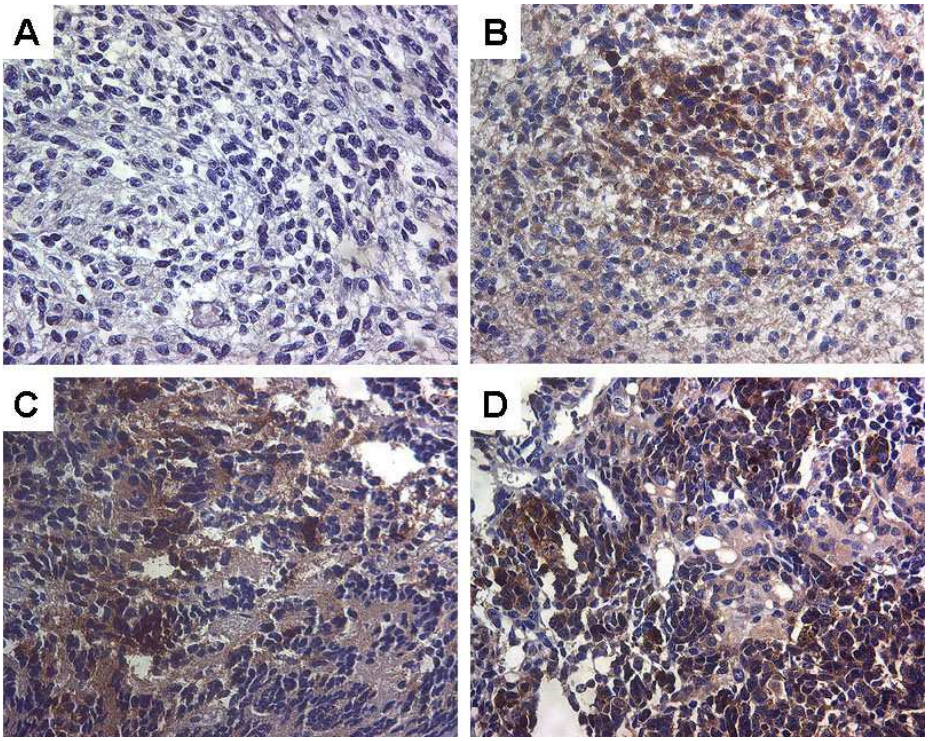
4. Apoptosis features and prognosis

The caspase 3 and Bcl-2 oncoproteins are studied as potent indicator of medulloblastoma prognosis. Immunoreactivity were caspase 3, 79% (46 of 58); and Bcl-2, 72% (42 of 58). The Bcl-2 labeling was diffusely reactive and some of the positive tumors were characterized by a weak, but clearly specific membrane staining of the tumor cell nucleus, others were strongly positive for Bcl-2 with the whole cell body stained (Fig 13) and caspase 3 positive cells displayed apoptotic morphology, such as condensed and fragmented nuclear chromatin (Fig 14). We were able to show that the apoptotic index is statistically significant associations between immunohistochemical variables studied as well as between clinical and pathological variables. The apoptotic index for Bcl-2 decreased from 14 patients (88%) to 2 patients (40%) and for caspase 3 from 11 patients (92%) to 7 patients (63%) that have found to be free of disease after the follow-up analysis. However elevated Bcl-2 from 2 patients (12.5%) to 3 patients (60%) and caspase 3 from 1 patient (8.3%) to 4 patients (36%) expressions was found to be related to short-term survival and apoptotic index with the p value of 0.04 and 0.05 respectively. Therefore Apoptotic index has a close association in prognosis of medulloblastoma patients but not specifically significant.



Immunoreactivity	Good prognosis	Poor prognosis
Negative (Fig A)	**14 (87.5%)**	**2 (12.5%)**
Positive	**33 (78.5%)**	**9 (21.4%)**
1+ (Fig B)	14	2
2+ (Fig C)	17	4
3+ (Fig D)	2	3

Fig 13. Immunoreactivity of apoptosis for medulloblastoma cells to Bcl-2. It is diffusely reactive and strongly positive within the whole cell body and membrane staining of nucleus. The scoring was negative, 1+, 2+, 3+ from (A) to (D) respectively (x 400) and p-value of 0.04.

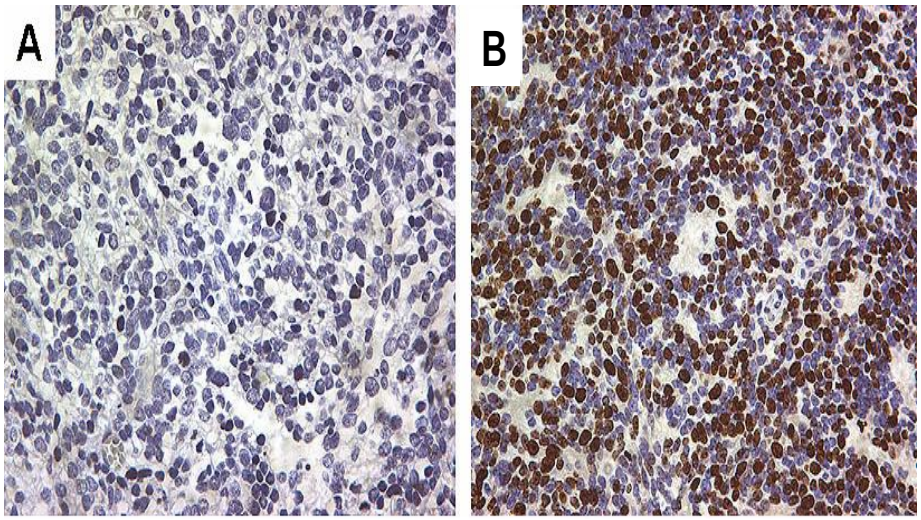


Immunoreactivity	Good prognosis	Poor prognosis
Negative (Fig A)	**11 (91.6%)**	**1 (8.3%)**
Positive	**36 (78.2%)**	**10 (21.7%)**
1+ (Fig B)	14	4
2+ (Fig C)	15	2
3+ (Fig D)	7	4

Fig 14. Immunoreactivity of apoptosis for medulloblastoma cells to caspase 3. It shows randomly distributed apoptotic cells with positive staining in medulloblastomas. The scoring was negative, 1+, 2+, 3+ from (A) to (D) respectively(x 400) and p- value of 0.05.

5. Proliferation features and prognosis

The proliferative marker of Ki-67 expressed randomly distributed and positively stained in nuclei (Fig 16). A variable proliferation capacity was observed in the different tumor structures. Neuronal, glial differentiation was few positive, while infiltration areas along penetrating vessels was strong positive. Proliferative index (PI) was calculated and averaged whereas the immunoreactivity of more than 20%, 31.5 ± 8.3% was defined as high PI, and less than 20%, 7.7 ± 10.2% as low PI. The number of cases with PI >20% was significantly greater in the group of tumors in patients with recurrent medulloblastoma as compared to those in patients with non-recurrent tumors. The prognosis was significantly poorer in high PI with p value of <0.01 and the positive correlation were also noted between AI and PI. Thus it is clear that the prognosis of medulloblastoma matters with PI.



Immunoreactivity	Good prognosis	Poor prognosis
Low proliferative index (<20%, 7.7±10.2%)	**26 (89.7%)**	**3 (10.3%)**
High proliferative index (>20%, 31.5±8.3%)	**21 (72.4%)**	**8 (27.6%)**

Fig 15. Immunoreactivity of medulloblastoma cells to proliferative marker of Ki-67 (x 400). The staining is randomly distributed and positively stained in nuclei which it A) exhibiting a low proliferative index of less than 20% of positive cells and B) more than 20% of strong positive cells showing high proliferative index (p-value of <0.01).

6. Oncogene & p53 features and prognosis

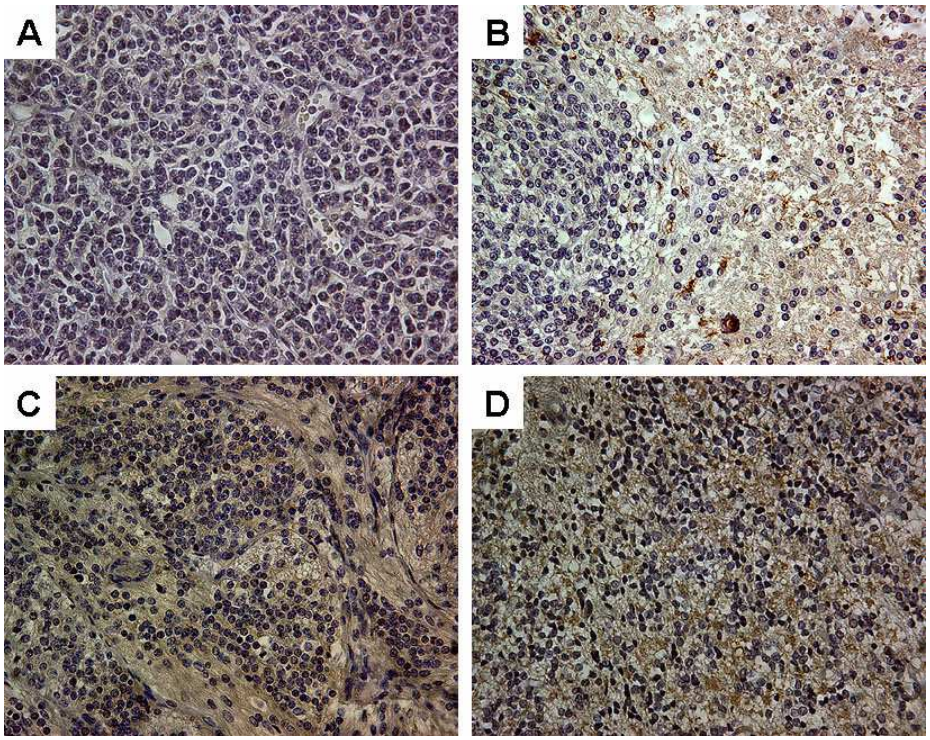
The prognostic variables of TrkC, 47% (27 of 58); and c-erB-3, 84% (49 of 58) are related to metastatic disease and represented the oncogene of medulloblastomas. TrkC is predominantly nodular, cytoplasmic and the positive tumor cells were distributed throughout the tumor in most of the cases (Fig 16) and c-erbB-3 was also cytoplasmic and well defined membrane (Fig 17). Expression of TrkC and c-erbB-3 in metastatic disease and prognosis are summarized in table 3 and of the clinical features considered, only metastatic disease at diagnosis was associated with a poor outcome. Age at the presentation and extent of resection were not predictive of prognosis or survival. Consequently the metastasis were also compared to prognosis and the values almost same in good or poor which it had no significant correlations among these medulloblastoma patients with p value of 0.63 and 0.57 respectively. The p53, 66% (38 of 58) reveals numerous cells to exhibit strong nuclear reactivity (Fig 18) and its immunoreactivity was the only biological marker predictive of a poor outcome in this assay. Therefore the expression was found to be related to short term survival with p value of 0.41 however the differences in numbers are small, hence it is not significant.

Table 3. Expression of TrkC and c-erbB-3 in metastatic disease and prognosis

Immunoreactivity	Good prognosis	Poor prognosis	Metastasis
Negative	**24 (77.4%)**	**7 (22.6%)**	**28 (48.2%)**
Positive	**23 (85.2%)**	**4 (14.8%)**	**27 (46.5%)**
1+	19 (86.4%)	3 (13.6%)	-
2+	3 (75%)	1 (25%)	-
3+	1 (100%)	0	-

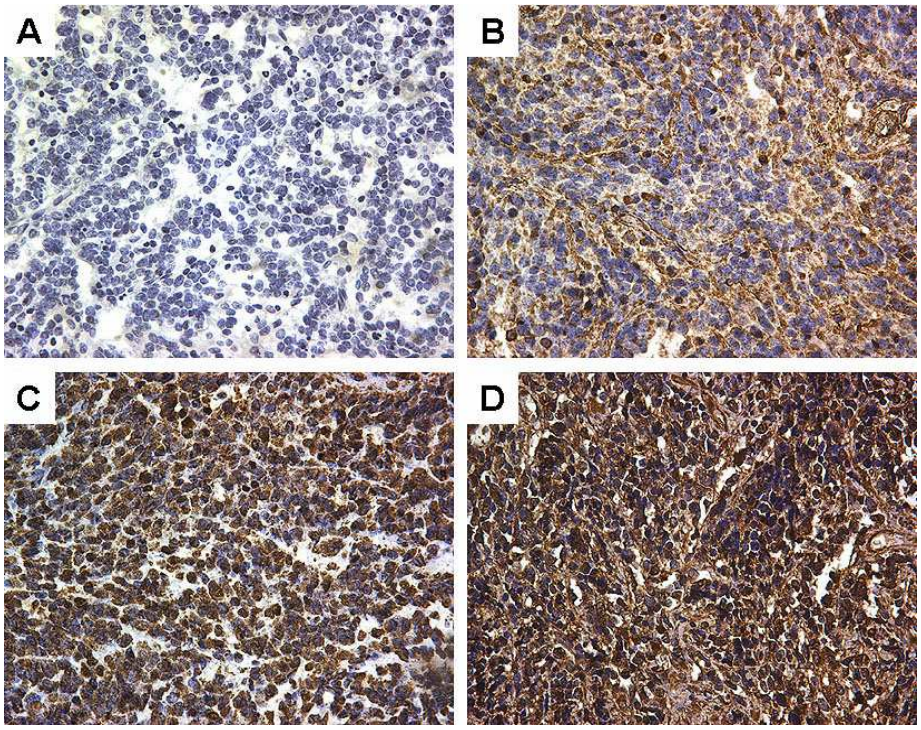
Immunoreactivity	Good prognosis	Poor prognosis	Metastasis
Negative	**8 (88.9%)**	**1 (11.1%)**	**28 (48.2%)**
Positive	**39 (79.6%)**	**10 (20.4%)**	**27 (46.5%)**
1+	23 (79.3%)	6 (20.7%)	-
2+	8 (72.7%)	3 (27.3%)	-
3+	8 (88.9%)	1 (11.1%)	-

** In table 3, three patients were of M unknown stage, therefore it was excluded from the statistical analysis. Top table; TrkC and bottom table; c-erbB-3.



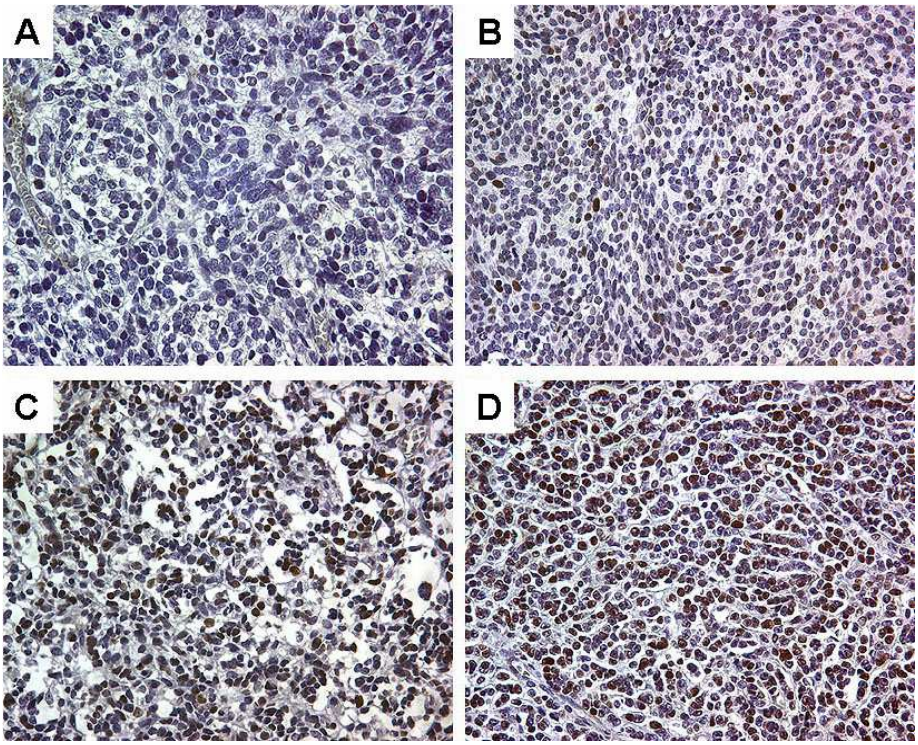
Immunoreactivity	Good prognosis	Poor prognosis	Metastasis
Negative (Fig A)	**24 (77.4%)**	**7 (22.6%)**	**28 (48.2%)**
Positive	**23 (85.2%)**	**4 (14.8%)**	**27 (46.5%)**
1+ (Fig B)	19 (86.4%)	3 (13.6%)	-
2+ (Fig C)	3 (75%)	1 (25%)	-
3+ (Fig D)	1 (100%)	0	-

Fig 16. Immunoreactivity of medulloblastoma cells to TrkC in metastasis and prognosis. It exhibited cytoplasmic staining and the scoring was negative, 1+, 2+, 3+ from (A) to (D) respectively (x 400) and p=0.63. .



Immunoreactivity	Good prognosis	Poor prognosis	Metastasis
Negative (Fig A)	**8 (88.9%)**	**1 (11.1%)**	**28 (48.2%)**
Positive	**39 (79.6%)**	**10 (20.4%)**	**27 (46.5%)**
1+ (Fig B)	23 (79.3%)	6 (20.7%)	-
2+ (Fig C)	8 (72.7%)	3 (27.3%)	-
3+ (Fig D)	8 (88.9%)	1 (11.1%)	-

Fig 17. Immunoreactivity of medulloblastoma cells to c-erbB-3 in metastasis and prognosis. It was well-defined cytoplasmic staining. The scoring was negative, 1+, 2+, 3+ from (A) to (D) respectively (x 400) and p=0.57.



Immunoreactivity	Good prognosis	Poor Prognosis
Negative (Fig A)	**18 (90%)**	**2 (10%)**
Positive	**29 (76.3%)**	**9 (23.7%)**
1+ (Fig B)	25	9
2+ (Fig C)	2	0
3+ (Fig D)	2	0

Fig 18. Immunoreactivity of medulloblastoma cells to tumor suppressor gene p53. It reveals numerous cells to exhibit strong nuclear reactivity. The scoring was negative, 1+, 2+, 3+ from (A) to (D) respectively (x 400) and p=0.41.

IV. DISCUSSION

Medulloblastoma is the most common malignant childhood brain tumor. It is a highly malignant neoplasm with a strong tendency to dissemination along the neuroaxis and formation of subarachnoidal and ependymal distant implants. Although some reports in the literature have shown up to a 70% 5 year survival for some of these patients, it is at the cost of significant long-term treatment related morbidity[30, 33].

The differences in clinical outcomes of medulloblastoma patients with similar tumor stages allow making suggestions about biological heterogeneity of these tumors. Therefore various immunohistochemical assays, so as proliferative markers, p53, caspase3, TrkC, c-erbB-3, and Bcl-2 oncoproteins, and others have been recently studied as potent indicators of medulloblastoma prognosis, but the results obtained are strongly heterogeneous[16, 17, 33-40]. All the same, a continued search for and verification of universally applicable biomarkers for medulloblastoma prognosis may contribute to the improvement of treatment protocols that still exist. It could also help to answer why medulloblastoma fail to respond to treatment and thereby indicate new routes for treatment development. However, previous studies have not examined multiple prognostic markers on the same set of tumors and

established their importance relative to clinical standards that already established. Therefore identification of the prognostic factors that distinguish patients at relatively low risk of tumor recurrence from those at high risk has important implications for treatment planning.

The present study, therefore discover the most appropriate prognostic markers which it manage the medulloblastoma that has been based on risk factors to develop the most accurate diagnosis and prediction of clinical outcomes. To overcome the obstacle of studying a large number of markers on a large number of tumors, we used tissue microarray technology and using this technology, it is possible to analyze multiple tumors with positive and negative controls on each slide. Therefore we made an attempt to evaluate the prognostic significance of thirteen immunohistochemical variables in the cohort of 58 medulloblastoma with standard and high clinical risk. All patients in our sample had undergone maximal surgical resection, craniospinal radiation therapy and chemotherapy. All three modalities are used in modern therapeutic protocols which have led to more than 60% of patients being cured of their disease[5] and M stage did not impact on overall survival or progression free survival.

In pediatric medulloblastomas, anaplasia defined as increased nuclear size and pleomorphism in large cells with dominant nucleoli in association with high

mitotic counts and apoptotic nuclei is a bad prognosticator[10, 14]. Increasing grades of anaplasia, particularly severe anaplasia, are associated with progressively worse clinical outcome and large cell medulloblastoma is recognized by large, round cells with single nucleoli and portend the worst prognosis[16]. However in our assay nuclear size and anaplasia did not predict any significance in prognosis of medulloblastoma.

Immunohistochemical methods have been used as a means of exploring the spectrum of histotypical markers expressed by medulloblastomas[26, 27]. GFAP and synaptophysin are markers of glial and neuronal differentiation, respectively. It has been theorized that a more differentiated tumor would have a more favorable prognosis. The prognostic values of GFAP and synaptophysin have been investigated in the past with conflicting results[23-26] and this study showed that there was no suggestions of a prognostic value of wither GFAP and synatophysin. Thus cell differentiation did not predict either of prognosis or overall survival of patients.

Apoptosis (programmed or suicidal cell death) occurs in numerous normal and pathological conditions, including neoplastic processes. It would be rationale to suppose that low levels of apoptosis are a poor prognostic pattern. In various neuroepithelial neoplasms, the apoptotic rate was associated with grade of tumor malignancy, but its prognostic value may be estimated as

controversial[28]. Concerning medulloblastoma, the results in this study obtained are also strongly heterogenous, although all studies showed large intertumoral and intratumoral variability in the apoptotic cell counts. Haslam et al[37] have investigated of 43 pediatric cases and they have found that patients with highest apoptotic index had significantly better clinical outcome. In contrast, Grotzer et al [41] have established that the apoptotic index does not predict clinical outcomes among the cohort from 78 PNET of CNS. The results of present study are differed significantly from those in two above mentioned reports. According to our findings, patients with lowest apoptotic index had better clinical outcome with p- value of 0.04 and 0.05. It is clearly that apoptotic cells in tumor tissue are the final product of a complex process of cell death and, therefore, many regulatory proteins may provide biologically important information.

The proliferative activity, of a tumor or tissue, is determined by the growth fraction and the time taken to complete the cell cycle. There is a strong correlation between the proliferation rate of tumors and clinical outcome[13-17]. The immunohistochemical expression of Ki67 is a good marker of positive cells generally correlates well with the histological malignancy and used to calculate proliferation index (PI). Its presence in large proportions of cells suggests an aggressive neoplasm. Indeed, it has been found to be a prognostic

indicator for poor outcome in some studies[13]. However our patient population confirmed that metastatic stage is a clinical factor with independent prognostic significance. To correct for the effect of clinical factors and to identify an independent prognostic role for tumor cell proliferation in medulloblastomas, metastatic stage and other clinical factors were included in all statistical models used. Therefore the analysis revealed that the value of PI had a significantly greater risk of progression and death in patients. Also a significant predictor or survival outcome in medulloblastoma as the value elevated with the p-value <0.01.

TrkC is a member of a family of three high-affinity neurotrophin receptor kinases and selectively binds neurotrophin 3. TrkC has been demonstrated to be a marker of good prognosis[12], although the study has been contradicted by an immunohistochemical analysis of 68 patients[33]. In our study, TrkC is not near significant marker of good or poor prognosis. Its effect was independent of the metastatic disease and other factors. The c-erbB-3 has been shown to a play a pivotal role in the signaling network formed by the epidermal growth factor receptor family and immunohistochemical studies and western blot analyses have suggested that it is a marker of poor prognosis in medulloblastoma[30, 42]. In our study, 84% of tumors were positive however the value of prognosis were not significant. In medulloblastoma any correlation

between apoptosis and its regulatory proteins' expression, so as p53 and bcl-2 have not been found yet. Therefore, the distinct regulatory mechanisms of apoptosis intensity in medulloblastoma are still unclear.

Therefore we conclude that this study, use known biological and clinical markers to model medulloblastoma risk and a technique that has been used successfully in other, similar tumors[30]. Although retrospective, we believe our study contributes substantially to the body of exiting knowledge and helps rationalize some of the apparent conflicts among the biological and clinical markers. Future efforts should be directed toward validating this model and approach in prospective studies.

V. CONCLUSION

We analyzed 58 patients with medulloblastoma using immunohistological techniques for several tumor factors in addition to clinical factors which it includes differentiation, large cell, anaplastic variant, proliferation index, oncogene and apoptosis of tumor cells. There was no significant correlation between the prognosis and the degree of cell differentiation. The positive correlation was noted between proliferative index and apoptotic index in a tumor mass. Therefore the results conclude that the proliferation index is directly linked to the prognostic factor for medulloblastoma and immunohistochemical staining is a potential powerful tool for predicting prognosis of medulloblastoma patients.

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ABSTRACT (IN KOREAN)

대규모 Tissue microarray와 면역조직화학염색을 이용한 수모세포종 환자의 예후 예측

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주 선 영

수모 세포종은 악성의 신경외배엽성 종양으로 소아에게 가장 빈도 높은 뇌종양 중 하나이다. 과거 20년간 수술 수기의 발달과 더불어 항암 요법 및 방사선 치료법의 발달로 수모 세포종 환아의 5년 생존률은 70-80% 까지 좋아지게 되었으나 치료 성적에 결정적으로 중요한 영향을 주는 방사선 치료가 장기간의 추적 관찰 결과 많은 후유증을 일으키는 것으로 알려졌다. 최근의 치료에 있어서의 주안점은 치료 성적의 손상 없이도 최소의 치료를 함으로써 장기 생존 환아의 후유증을 최소화 하자는 것이다. 따라서 환자의 예후를 짐작하는 것은 치료의 강도를 정하기 위해 아주 중요하며, 또한 환자의 임상적인 정보는 예후 예측에 아주 중요한 역할을 한다. 하지만 임상적 예후 인자만을 판단하여 예후가 좋을 것으로 생각되는 환자에서도 많은 경우의 예외를 관찰해 왔으며, 이런 예외의 경우에 무엇이 그 예후 인자인가에 대한 많은 연구가 있었다. 특히, 종양 세포

자체의 여러 가지 생물학적 특성이 환자의 예후와 관련 있을 것이라는 주장과 병리 조직 소견상 종양의 분화 형태에 따른 예후가 차이가 있을 수 있다는 보고도 있었다.

본 논문은 임상적으로 위험도가 같은 임상적 예후 인자를 가진 수모 세포종 환자에서 종양세포 자체의 생물학적 특성만으로 예후의 차이가 있는지 확인하고자 한다. 3세 이상으로 일정한 항암요법과 방사선 치료를 마치고 1년 이상 추적 관찰 되었던 58명의 환자를 분석하고 수술 중 채취한 이들의 파라핀 고정 조직을 이용하여 병리학적 특성과, tissue microarray를 이용하여 종양의 분화, 증식능과 사멸능, 그리고 oncogenesis 등을 비교함으로 예후간의 상관관계를 분석하였다. 분석 결과 종양의 분화 양상에 따라 여러 종류의 항원이 발현 되었지만 분화형과 예후간의 상관관계는 나타나지 않았지만 종양증식이 증가할수록 세포자멸사가 증가함을 알 수 있었으며, 종양의 증식능이 20% 보다 높은 환자들은 재발의 확률도 높았다. 또한 분화에 따른 증식능과 사멸능의 변화를 관찰 한 결과 예후 예측인자로서 증식능이 환자의 예후를 판단하고 향후 치료를 결정할 수 있는 중요한 지표임을 알 수 있었다.

핵심되는 말: 증식능, 사멸능, 수모 세포종, 예후인자, 세포자멸사