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Histopathological Study and Immunohistochemical Expression of WT1 Protein in Different Types and Grades of Astrocytic Tumors

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Abstract: Wilms' tumor gene product (WT1) is an embryonic zinc-finger transcription factor, which was originally identified as a tumor suppressor gene and documented as a target for gene therapy of many solid tumors including astrocytic tumors. The aim of this study was the assessment of immunohistochemical expression of WT1 protein, in different histologic types and grades of astrocytic tumors. WT1 protein expression will be correlated with various clinical data (such as age, sex). The material of this study consisted of 80 paraffin blocks of astrocytoma cases collected from Nasser Institute for Research and Treatment from January 2012 to December 2013. Results revealed that WT1 was expressed in (25/28) grade I astrocytomas, all grade II astrocytomas (14/14), (13/16) grade III astrocytomas and (21/22) grade IV with highly significant relationship (P-value <0.001) between grade I and grade IV, grade III and grade IV. Expression was; the highest in older ages (significant p-value = 0.034), in male cases (insignificant p-value = 0.298) and in areas of perivascular proliferation and the astrocytic tumors subtypes (gemistocytic astrocytoma, pleomorphic xanthoastrocytoma, protoplasmic astrocytoma). In Conclusion: Many factors affected the expression of WT1 in astrocytomas rather than the tumor grade, which included tumor vasculature, patient age and the tumor subtype. Further studies are recommended for assessment of WT1 expression in astrocytomas and correlation of WT1 expression with the extent of tumor vasculature and each of astrocytoma histologic subtype and patients' age.

Key words: Astrocytoma · Target therapy · WT1 Protein

INTRODUCTION

Astrocytomas are CNS neoplasms in which the predominant cell type is derived from an immortalized astrocyte [1]. Astrocytic tumors were divided into four grades according to difference in their location in the central nervous system (CNS), morphologic features and progressive and invasive behaviors.

Wilms' tumor (WT1) gene is the gene that encodes a zinc finger transcription factor playing an important role in cell growth and differentiation. WT1 was first identified as a tumor suppressor gene while the wild type was proved to be an oncogene [2].

The WT1 gene is highly expressed in the majority of acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) [4]. It is also expressed at high levels in almost all types of solid tumors including breast, ovarian and astrocytic tumors [5]. WT1 is an exciting target for therapy because it can regulate many aspects of tumorigenesis; further, its expression in the normal brain is essentially absent [6].

The aim of this study was the assessment of immunohistochemical expression of WT1 protein in different grades and histologic types of astrocytic tumors and the expression was correlated with various clinical data (such as age, sex).

MATERIAL AND METHODS

Study Design: A retrospective cross-sectional study was carried out. The material of this study consisted of 80 paraffin blocks of astrocytoma cases collected from archives of Pathology Department of *Nasser Institute for Research and Treatment* from "January 2012 to December

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Table 1: Histologic subtypes and clinicopathologic features of the studied astrocytomas:

WHO Grade [2]	Astrocytoma Subtype	No. of cases	Age		Sex	
			Mean	Range	Male	Female
I	• Pilocytic Astrocytoma	27	11.7	4-25	14	13
	• Subependymal Giant Cell Astrocytoma"SEGA"	1	6	-----	0	1
II	• Fibrillary Astrocytoma	7	42.1	28-51	6	1
	• Gemistocytic Astrocytoma	3	33	30-38	2	1
	• Protoplasmic Astrocytoma	1	49	----	1	0
	• Pleomorphic xanthoastrocytoma "PXA"	3	26	11-55	3	0
III	Anaplastic Astrocytoma	16	42.4	23-56	12	4
IV	Glioblastoma	22	53.5	10-76	15	7
	Total	80	32.2	4-76	53	27

2013" after exclusion of the cases with unsatisfactory tissue material. The clinical data were obtained from the pathology reports of the patients. These data included age and gender (Table 1) and radiologic location.

Histologic Review: Each paraffin block was re-cut by rotatory microtome at 5 microns thickness then mounted on glass slides to be stained by haematoxlyin and eosin (H and E) for histopathological re-evaluation. The cases were classified according to WHO Classification of astrocytic tumors into grades I, II, III, IV.

Immunohistochemistry Steps: Paraffin embedded sections were made at 4 microns thickness and mounted on positive charged slides. Positive control slides were used in the same run (mesothelioma). Immune-staining for WT1 (DAKO, clone 6F-H2) was done for all cases by Bench Mark IHC/ISH staining module (Ventana) and the steps occurred automatically including De-paraffinization, Cell conditioning (Standard CC1 application) for 80 minutes, Application of one drop (100µ) of the antibody, Application of cover slip and incubation for 32 minutes, Application of one drop of DAB (counterstain) with Haematoxylin and incubation for 8 minutes. The slides were extracted and arranged in racks, washed in tap water and soap for 5 minutes, dehydrated in the ascending grades of alcohol for 5 minutes in each container and slides were cleared in xyline and then cover slips were applied.

Interpretation of WT1 Immune-Staining: Assessment of immune-staining was performed using Olympus light microscope (CX34). Sections were examined for expression of WT1 was identified as cytoplasmic staining in tumor cells using Bassam *et al.* [7] scoring system. Frequency of WT1 expression by the tumor cells was scored as (0) {0 %}, (1) {<25%}, (2) {25-75%}, (3) {>75%}. Intensity of WT1 staining by the tumor cells score was considered as score (0) for {negative expression}, score (1) for {mild intensity compared to normal glial cells}, score (2) for {moderate intensity intermediate between mild and marked intensities}, score (3) for {marked intensity compared to normal glial cells}. WT1 index was determined as the sum of frequency and intensity scores giving us six indices (0, 1, 2, 3, 4, 5 and 6). The six indices were grouped as, negative (0 index), mild (1 and 2 indices), moderate (3 and 4 indices), marked (5 and 6 indices).

Statistical Analysis: Data was analyzed using IBM SPSS advanced statistics version 20 (SPSS Inc., Chicago, IL). Numerical data were expressed as mean and standard deviation or median and range as appropriate. Qualitative data were expressed as frequency and percentage. Chi-square test or Fisher's exact test was used to examine the relation between qualitative variables. For not normally distributed quantitative data, comparison between two groups was done using Mann-Whitney test (non-parametric t-test). Comparison between 3 groups was done using Kruskal-Wallis test (non-parametric ANOVA) then post-Hoc "Scheffe test" was used for pair-wise comparison based on Kruskal-Wallis distribution. Spearman-rho method was used to test correlation between numerical variables. All tests were two-tailed. A p-value < 0.05 was considered significant.

RESULTS

Clinicopathologic Results: The material of this study consisted of 80 paraffin blocks of astrocytoma cases. In this study, male patients represented the majority of cases being 53 (66.3%) cases while female patients were 27 (33.8%) with a ratio (1.9/1). The mean age for the cases enrolled in the present study was (32.2 +/- 19.8 years) and

Table 2: Correlation between WT1 expression in astrocytomas, tumor grade and clinicopathologic aspects:

NO. of cases with WT1 expression	WHO Grade GI	GII	GIII	GIV	Age Mean (Range)	Sex Male	Female
Negative	3	0	3	1	25.9 (7-49)	2	5
Mild	6	5	10	0	37.4 (8-56)	14	7
Moderate	14	3	2	2	22.3 (4-65)	15	6
Marked	5	6	1	19	40.7 (4-76)	22	9
	P-Value					0.034	0.298

Table 3: Multivariate analysis of WT1 expression in different astrocytoma grades

Grade	Grade	P-value
G I	G II	0.555
	G III	0.116
	G IV	< 0.001
G II	G III	0.012
	G IV	0.032
G III	G IV	< 0.001

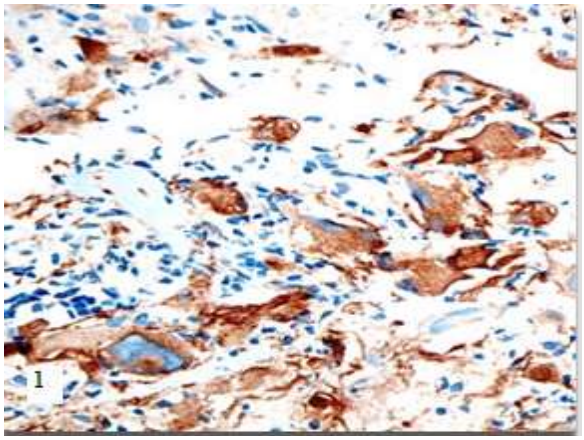
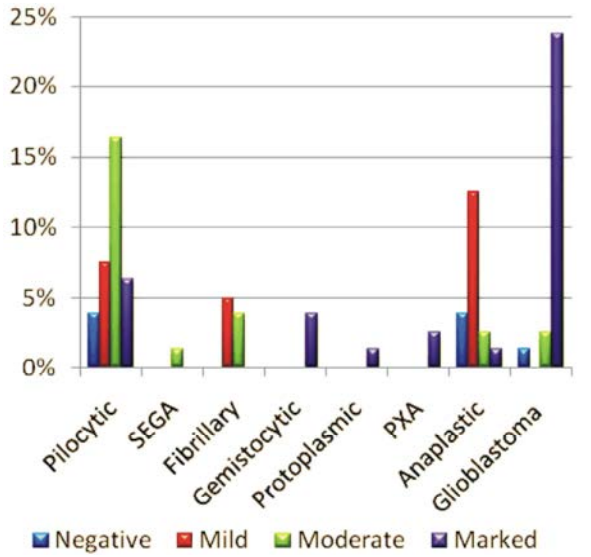


Fig. 1: WT1 moderate immunohistochemical cytoplasmic expression in pleomorphic xanthoastrocytoma"Note the intranuclear cytoplasmic inclusions'(x200).

ranged between (4-76 years old). Most of the included cases were located in the parietal lobe (19 cases) (38.8%) followed by occipital lobe which was involved by 18 (27.5%) cases and frontal and temporal lobes being involved by {14 (17.5%) and 13 (16.3%)} respectively. The cerebellum was involved by 8 (10%) cases. Three cases (3.8%) showed ventricular masses while two cases (2.6%) presented with sellar and suprasellar astrocytomas. The least number of astrocytomas was seen in corpus callosum, optic nerve and thalamus {single case (1.3%) for each}.



Graph 1: WT1 expression in different astrocytoma subtypes:

The studied cases were classified according into 28 (*35*%) cases of grade I astrocytomas, 14 (*17.5*%) cases of grade II astrocytomas, 16 (*20*%) grade III astrocytomas and 22 (27.5%) grade IV astrocytoma (Table??). The histologic subtypes were illustrated in Table (1). The sixteen anaplastic (grade III) astrocytomas included 4 cases (25%) showing associated gemistocytic features and one case (6.25%) with pleomorphic xanthoastrocytoma features.

Immunohistochemistry: WT1 expression in the current study was detected in 73 (91.2%) out of 80 cases being seen in (25/28) grade I astrocytomas, all grade II astrocytomas (14/14), (13/16) grade III astrocytomas and (21/22) glioblastoma with significant relationship between grade I and grade V and grade III and grade IV (p-value <0.05) (Table 3). Highest indices of WT1 expression were seen in glioblastoma, gemistocytic, the majority of PXA's and the only included protoplasmic astrocytoma case {19 (23.8%), 3 (3.8%), 2 (2.5%), 1 (1.3%) respectively} while

Table 4: Correlation between WT1 expression in different grades of astrocytomas and age and sex:

		Age		Sex	
WT1	Grade	Mean	Range	Male	Female
Negative	GI	11.3	(7-16)	0	3
	GII	0	0	0	0
	GIII	33.7	(28-37)	1	2
	GIV	46	---	1	0
Mild	GI	16	(8-25)	2	4
	GII	40.4	(10-55)	4	1
	GIII	48.7	(37-56)	8	2
	GIV	0	0	0	0
Moderate	GI	11.3	(4-22)	8	6
	GII	49.5	(49-51)	3	0
	GIII	34	(30-38)	2	0
	GIV	50	(35-65)	2	0
Marked	GI	8	(4-14)	3	2
	GII	48.5	(11-49)	5	1
	GIII	23	---	1	0
	GIV	52.8	(28-66)	13	6

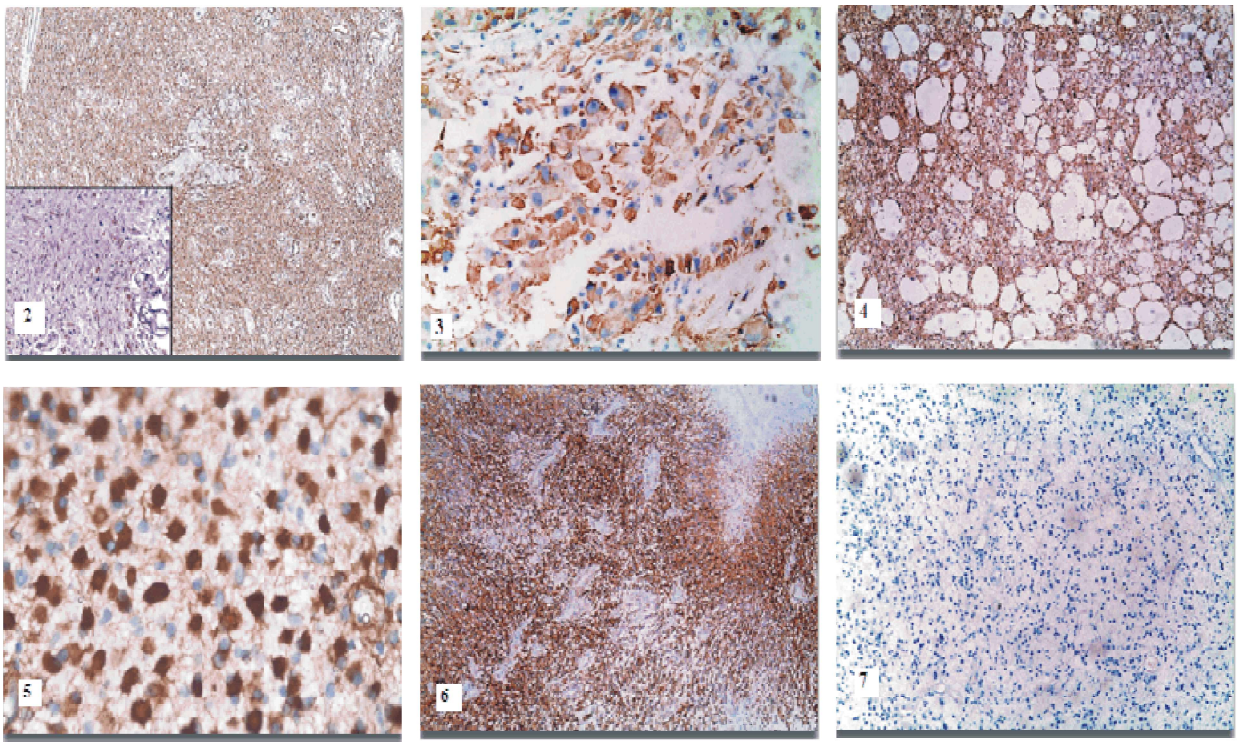


Fig. 2-7: Immunohistochemical expression of WT1 in different astrocytic tumors. 2) Marked WT1 cytoplasmic expression in pilocytic astrocytoma, note the extensive vascular proliferation) (x50). 3) Moderate cytoplasmic expression, Sub ependymal Giant Cell Astrocytoma(x100).
4) Marked cytoplasmic WT1 expression in protoplasmic astrocytoma(x50).
5) WT1 marked cytoplasmic expression in Gemistocytic Astrocytoma (x200). 6) WT1 marked cytoplasmic expression in glioblastoma, note the strong immunoreactivity in the glial cells around the palisading necrosis (x50). 7) Anaplastic Astrocytoma with WT1 negative cytoplasmic expression(x50).

most of anaplastic and fibrillary astrocytomas {10 (12.5%), 4 (5%)} showed mild expression. The only SEGA case and the majority of pilocytic cases showed moderate expression (Graph 1). The only three anaplastic cases (18.8%) showing higher expressions showed associated gemistocytic and pleomorphic xanthoastrocytoma histologic features. WT1 expression was higher in elder ages {significant *P-Value* (< 0.05)} and male patients {insignificant *p- value (> 0.05)*} (Table 2). Negative expression was seen in females with grade I and grade III astrocytomas but only noticed in males presenting with grade III and grade IV astrocytoma. The only glioblastoma case showing negative expression was the youngest among the presented grade IV cases (Table 4).

DISCUSSION

All astrocytomas expressing WT1 showed a cytoplasmic staining including delicate cell processes. WT1 in Wilms' tumors is predominantly observed in the nucleus, however it has been reported that WT1 protein is able to shuttle from the nucleus to the cytoplasm and a significant proportion of cytoplasmic WT1 protein is associated with ribonucleoprotein particles (RNPs) in mouse mesonephros-derived M15 cells, suggesting that WT1 is involved in RNA metabolism [8]. Heterogeneous protein expression seen in many samples may be due to cell cycle related changes in WT1 expression, as it has been proved that WT1 protein is involved in cancer cell proliferation by regulating cyclin D1 protein levels [9].

Maximum immunostaining in all positive cases was seen in highly cellular portions with high vascularity which agrees with Hashiba *et al*. [10], who found that WT1 protein was strongly expressed in the anaplastic portions and areas with perivascular proliferation, indicating that WT1 gene might be important in glial tumor cell proliferation.

Higher expressions detected in current grade I astrocytomas may be attributed to the marked vascular proliferation histologically observed in the studied cases. That issue was explained in Bassam *et al*. study [7], by the positive relationship between the Mean Vascular Density (MVD) and the high WT1 expression. McCarty *et al.* [11], also found a correlation between levels of WT1 expression and VEGF expression in Ewing's sarcoma cell lines and that WT1 plays a key role in optimizing the response of tumor cells to hypoxia. Hashiba *et al.* [10], stated high WT1 expression in the studied sub ependymal giant cell astrocytomas and that copes with our findings.

In consistence with our results, negative expression was recorded in grade II astrocytomas enrolled in Hashiba *et al.* 10], meanwhile it was absent in Parvin and Zahra [12], who stated moderate and mild WT1 expression in the studied grade II cases. Gemistocytic astrocytomas reported in Schittenhelm *et al.* [13], showed marked expression, while fibrillary astrocytomas presented in Bassam *et al.* study [7] revealed domination of moderate expression and all studied pleomorphic xanthoastrocytomas expressed WT1 in Schittenhelm *et al.* study [15], keeping with current results.

Contradictory to present results, most of anaplastic astrocytomas showed marked expression in Parvin and Zahra study [12]. Also, Hashiba *et al*. [10] recorded high scores in anaplastic cases. Negative expression in anaplastic astrocytomas enrolled in this study was attributed to IDH1 mutation in young ages with high grades astrocytoma explained by Rauscher *et al*. [14] who reported that grade II cases positively expressed WT1 in older patients than those who negatively expressed WT1, meanwhile negative expression in anaplastic astrocytomas and glioblastomas was seen in young patients compared to positive expression in the elder age in the same grades cases. However, grade I cases in the fore mentioned study revealed nearly similar ages of cases that positively and negatively expressed WT1.

Selective expression of WT1 in certain glioblastoma cell lines rather than the others, recorded by Yusuke *et al*. [16] has explained the negative expression seen in a single case in the present study. On the other side, Bassam *et al.* [7], recorded absence of negative expression in the studied glioblastoma cases and mild expression in a single case.

CONCLUSIONS

Many factors affect the expression of WT1 in astrocytomas rather than the tumor grade which include tumor vasculature, patient age and tumor subtype.

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