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WT1 Expression in Glial Tumors: Its Possible Role in Angiogenesis and Prognosis

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Abstract: WT1 gene has oncogenic properties and its continuous over-expression is related to leukemia and several kinds of solid malignancies. Growth of WT1-expressing leukemia and solid tumor cells could be inhibited by the treatment with WT1 antisense oligomers. Various studies revealed WT1 expression in a considerable proportion of glial tumors and related it to high grade tumors and proliferative activity. Our study aimed to evaluate expression of WT1gene in glial tumors and to correlate its expression with various clinicopathological parameters and tumor angiogenesis to include or exclude WT1 as a potential prognostic factor and therapeutic target in patients with glial tumors. 50 cases of glial tumors were immunohistochemically evaluated for WT1 expression in tumor cells and for CD34 expression in intra-tumoral endothelial cells to calculate MVD. We correlated WT1 expression with tumor grade, MVD, mitotic count, tumoral necrosis and age of patients. Evaluation of WT1 immunostained sections revealed positive staining in 43 cases (86%). WT1 staining in all cases was cytoplasmic with five cases (10%) showing concomitant nuclear expression. Statistical analysis revealed significant differences between mean WT1 score among different tumor grades, strongly positive but yet non-significant correlation between mean WT1 score and each of mean MVD and mean mitotic activity, for all tumor grade groups and non-significant correlation with age of patients or extent of necrosis in grade IV tumors. The current study concluded that WT1 is expressed in a considerable percent of glial tumors and bears relation to tumor grade and thus prognosis and bears strongly a possible but yet not well established correlation with tumor angiogenesis and proliferative activity. This makes it a useful marker for prognosis and possible target for cancer therapy. However we must carry out further studies to confirm such role, declare the mechanism, which is probably related to downstream target genes. The scoring of WT1 expression is also not standardized between researches and we recommend it to be either negative (score 0), weak (scores 1-2), moderate (scores 3-4), or strong (scores 5-6) and consider positivity from scores 3 to 6 and negativity from scores 0 to 2.

Key words: Angiogenesis · Glial tumors · Prognosis · WT1

INTRODUCTION

Max Wilms (1867-1918) was a German surgeon who studied pediatric kidney tumors and a thorough review over his findings, *Die Mischgescwülste der Niere* was published in 1899. A certain kind of kidney cancer in children was named as Wilms' tumor. In 1990, two different research groups cloned the gene linked to a specific mutation, a deletion of chromosome region 11p13 found in 10% of the Wilms' tumor cases [1] and accordingly, the identified gene was named Wilms' tumor gene 1 [2]. Wilms' tumor gene 1 (WT1) spans 50 kb genomic DNA and consists of 10 exons which encodes an mRNA transcript of about 3.2 kb. The mRNA translates into a 429 amino acid protein with proline and glutamine rich amino terminus harboring defined functional domains exerting transcriptional repression, activation, self-association, RNA-recognition and nuclear localization signals. The carboxyl terminal part of WT1 contains four Cys2His2 zinc-fingers, encoded by exon 7-10, conferring specific DNA-binding. Due to alternative splicing of the WT1 pre-mRNA there are four major isoforms: WT1 (-17AA/-KTS), WT1 (+17AA/-KTS), WT1 (-17AA/+KTS) and WT1 (+17AA/+KTS) [3]. The first alternative splicing

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event affects the entire exon 5 and leads to presence or absence of 17 amino acids (17AA). The second alternative splicing event generates an insertion/no insertion of three amino acids, lysine, threonine, serine (KTS), in the very end of exon nine, affecting the conformation of zinc finger three and four in the WT1 protein [4].

A new isoform, termed sWT1 due to its small size (35-37 kDa), was identified a few years ago. A second promoter, located in intron one generates mRNA that codes for the sWT1 protein, which lacks the repression domain found in the N-terminus of the full-length protein. The sWT1 was reported to be overrepresented in leukemic samples and to confer oncogenic properties due to strong activation of certain WT1 target genes, as compared to full-length WT1 [5]. The proximal promoter of the WT1 gene is transcriptionally active in several cell lines, independent of WT1 expression and therefore tissue-specific expression of the WT1 gene must depend on additional regulatory elements [6].The mechanisms for tissue-specific transcriptional regulation of WT1 are to a large extent unexplored [2]. The hematopoietic globin transcription factor 1, GATA-1, has been implied in the regulation of WT1 expression in hematopoietic cells [7]. In acute myeloid leukemia a correlation of PAX2 levels with WT1 expression has been observed, which suggests PAX2 to be a WT1-activator in a subset of AMLs [8]. Pea3 (polyomavirus enhancer activator 3), a transcription factor co-expressed with WT1 in the developing kidney transactivates the WT1 promoter [9]. Other transcription factors reported to affect WT1 expression include Sp117, WT118 (negative autoregulation), PAX819 and nuclear factor kappa B, NF [10]. The WT1 protein has been proven to interact with the modifying E2-ligase, ubiquitin-conjugating enzyme 9 (Ubc9), now known as a SUMO-1 E2-ligase [11]. SUMO is an ubiquitin related protein, which is covalently attached to specific target proteins, resulting in alteration of their interaction with other proteins, altered localization and stability [12].

The WT1 protein is a transcription factor with high nucleotide conservation among species [13]. The ratio between the +KTS/-KTS isoforms is critical for proper gonadal and renal development and disturbance of this ratio causes intersex disorders. Specific mutations within the zinc-finger region of the WT1 protein lead to dysregulated sexual and renal development as seen in Denys-Drash syndrome (DDS) [14]. Its mutation and inactivation was seen in a subset of Wilms' tumor and children who were genetically predisposed to kidney neoplasm [15]. The WT1 gene was originally isolated as a tumor suppressor gene responsible for Wilms' tumor [16]. Further research showed that wild-type WT1 has oncogenic properties and its continuous over-expression is related to leukemia [17] and several kinds of solid malignancies including lung [18], colon [19], breast [20], thyroid cancer [21], astrocytic tumors [15], pancreatic ductal cancer [22] and bone and soft tissue sarcoma [23]. Moreover, high expression levels of WT1 mRNA correlated with poor prognosis in leukemia [17], breast cancer [20] and soft-tissue sarcoma [24] and with high tumor-stage in testicular germ-cell tumors [25]. How WT1 contributes to tumor growth and influences prognosis remains unclear. The role of WT1 in tumor genesis and the mechanism by which expression affects prognosis are both unclear; however, because the WT1 protein has transcriptional regulatory activity, effects on tumor genesis and prognosis are probably related to altered expression of key WT1 target genes [26]. Growth of WT1-expressing leukemia and solid tumor cells was inhibited by the treatment with WT1 antisense oligomers [22]. Hashiba *et al*. [27] reported that WT1 protein was detected in 70 of the 73 glial tumors (95.9%) examined. Almost all glioblastomas, anaplastic astrocytomas, anaplastic ependymomas and anaplastic oligodendrogliomas expressed high levels of WT1 protein. A significant correlation was found between WT1 protein expression and MIB-1 staining index. Histological examination found that WT1 protein was strongly expressed in the anaplastic portions and areas with perivascular proliferation and high cellularity, implying that WT1 gene might be important in glial tumor cell proliferation. They indicated that many malignant glial tumors are good candidates for cancer immunotherapy targeting WT1 protein and that WT1 protein expression could be used as a proliferation marker in glial tumors.

The presence of WT1 in glioma cell lines and the majority of primary tumor samples and its absence in normal astrocytes support the suggestion that WT1 expression is important in glioma biology [28]. McCarty *et al*. [26] demonstrated that WT1 up-regulates VEGF transcription, resulting in increased angiogenic activity, in Ewing sarcoma cell lines, they found a correlation between endogenous WT1 expression and VEGF expression. Up-regulation of WT1 in the low WT1-expressing cell lines led to a corresponding increase in VEGF expression (both mRNA and protein), whereas silencing of WT1 in the high WT1-expressing cell lines led to a corresponding decrease in VEGF expression. They demonstrated, from promoter-reporter, chromatin immunoprecipitation and site-directed mutagenesis assays, that VEGF is a direct target of the transcriptional

regulatory activity of WT1**.** WT1 has also been found to be expressed in the endothelial cells of tumors, consistent with its role during development where it significantly contributes to the normal vascularization of the embryonic heart [29]. 113 tumor specimens from different tissue types were analyzed and WT1 protein was found to be expressed in the vascular endothelial cells of 108. WT1 was also shown to increase the proliferation and migration of endothelial cells by regulating the levels of the Ets-1 transcription factor in these endothelial cells [30].

Our study aims to evaluate the potential expression of WT1gene in glial tumors and to correlate its expression with various clinicopathological parameters that might influence prognosis as tumor grade, mitosis, necrosis and age. Our study also aims to correlate WT1expression with tumor angiogenesis as detected by micro vessel density (MVD). This will help us to include or exclude WT1 as a potential prognostic factor and therapeutic target in patients with glial tumors and to detect a potential role in vascular tumoral proliferation.

MATERIALS AND METHODS

Study Design: Retrospective cross-sectional study. Paraffin blocks with satisfactory material of 50 cases diagnosed as glial tumors, were collected from archives of Pathology Department, Faculty of Medicine, Cairo University and a private laboratory, during the time period from January 2010 up to December 2012. Samples with unsatisfactory amount of tissue were excluded. Age and sex of each case were registered from pathology reports. Three serial sections from each tumor tissue block were cut at 5 micron thickness. One section was mounted on a glass slide then stained with routine Haematoxylin and Eosin stain (H&E) for histopathological evaluation. The other two sections were mounted on charged sides then immunostained with anti CD34 antibody and anti WT1 antibody. Each H&E tissue section was revised for tumor type, grade in accord to WHO criteria 2007 [31], extent of necrosis expressed as percent to section area and mitotic count per 10 HPFs (x400) were recorded.

Steps of Immunohistochemical Staining: For deparafinization and antigen retrieval, sections were heated in dry oven at 60°C for 30 min and then immersed in xylene, alcohol and finally 10 mM citrate buffer (pH»6). Endogenous peroxidase activity was reduced with 0.3% H_2O_2 solution. Sections were incubated with anti-human WT1 antibody (6F-H2, DAKO, U.S.A.; diluted 1:40) for 1 h and then processed with EnVisionä polymer. Immunorecative cells were visualized by immersing in Diaminobenzidine (DAB) for 5 min and then haematoxylin for 2 min as a counter stain. Samples were washed after each step by Phosphate Buffered Saline (PBS). For CD34 immunostaining, similar sections were incubated with anti CD34antibody (Clone QBEnd-10, Code M7165, DAKO Denmark; diluted at 1:25) for 30 min. Other steps were performed on the same serial steps used for WT1 immunohistochemistry, except for using Tris-EDTA buffer (ph9.9) instead of citrate buffer.

Interpretation of WT1 Immunostaining: All specimens were observed under light microscope. Only samples in which endothelial cells showed WT1 expressions were included the study, which were utilized as internal positive control and normal glial tissue were utilized as internal negative control. WT1 index was determined as the sum of WT1-positive relative frequency score and WT1 staining score.WT1 staining score was considered as 0 (negative: no staining), 1 (weak: mildly increased staining compared to the normal glial cells), 2 (intermediate: staining at intensity between 1 and 3) and 3 (strong: significantly increased staining in tumor cells compared with normal glial cells). Relative frequency of WT1-positive tumor cells was scored as 0 (0%), 1 (<25%), 2 (25-75%) and 3 (>75%) [15]. In addition the distribution of WTI protein expression was evaluated in the whole tissue section and also intra-cellular (cytoplasmic-nuclear).

Interpretation of CD34 Immunostaining*:* The endothelial cells of the proliferating vessels show cell surface membrane immunoreactivity. The numbers of positively stained intra-tumoral microvessels were counted per 10 high power fields (magnification x 400) and the count was reported as micro vessel density (MVD) [32]. Though WT1 stains endothelial cells, but we can't rely on it to calculate MVD, as the positive staining of the surrounding glial cells makes it difficult to highlight the micro-vascular spaces which may be considered as artifactual clefts.

Statistical Analysis: Results of histopathological and immunohistochemical assessment will be collected and tabulated, then analyzed using SPSS version 20 program using descriptive data analysis, Spearman correlation coefficient test and general linear model (GLM) repeated measures; MANOVA test (Test of within subjects effect); to find a possible correlation between expression of WT1 and MVD, tumor grade, percent of necrosis, mitosis and

age. P value less than 0.05 will be statistically significant.

RESULTS

The current study included 50 cases of glial tumors. 29 (58%) patients were females and 21 (42%) were males, with female to male ratio 1.38: 1. The ages of patients ranged from 2 up to 71 years with a median age of 34.85 (±21.1) years. 43 (86%) cases were astrocytic tumors, 5 (10%) cases were ependymal, one case (2%) was oligodendrocytic and one case (2%) was a mixed glioma; astro-oligodendrocytic tumor. The various histological subtypes and grades are illustrated in Table 1. Histopathological examination of H& E slides was conducted to revise tumor type and grade, then the mitotic count per 10 HPFs (x400) in all tumors were recorded and the mean mitotic activity for each tumor grade group was calculated and illustrated in Table 2. Statistical analysis using MANOVA; revealed significant differences between means of mitotic activity among different tumor grades, with p-value <0.05 (Test of within subjects effect), emphasizing the fact that mitotic activity increases with increasing tumor grade. Micro vessel density (MVD) was estimated for each tumor by counting the sum of intra-tumoral microvessels per 10 HPFs (x400) with positive cell membrane staining for CD34 (Figure; 1e) and then the mean MVD for each tumor grade group was calculated as shown in Table 2. Statistical analysis using MANOVA; revealed significant differences between MVD means among different tumor grades, with p-value <0.05 (Test of within subjects effect), emphasizing the fact that MVD increases with increasing tumor grade.

Evaluation of WT1 immunostained sections revealed positive staining in 43 cases (86%), ranged from score 2 up to score 6 as illustrated in Table 2 and Figs 1a-d, 1f. WT1 staining in all cases was cytoplasmic with five cases (10%) showing concomitant cytoplasmic and nuclear expression. Maximum immunostaining in all positive cases were seen in highly cellular portions with high vascularity. Among negative WT1 staining, score 0; all cases were low grade tumors; I and II; 7 out of 7 (100%). Among high WT scores; 5&6; grade III and IV (high grade) tumors constituted the majority, 8 (88.8%) out of 9 in score 5 and 9 (100%) out of 9 in score 6. Means of WT1 scores for each tumor grade were calculated (Table 2) and statistical analysis MANOVA; revealed significant differences between means among different tumor grades, with p-value <0.05 (Test of within subjects effect), emphasizing the fact that WT1 score increases significantly with increasing tumor grade. Statistical analysis using Spearman correlation to detect relation between WT1 score and MVD revealed non-significant

Table 1: The various histological subtypes and grades for glial tumors included in the study.

Item	Number	WHO grade	Histological Subtype	Number	Percent to total
Astrocytic tumors	43	I	Pilocytic	11	22%
			SEGA	2	4%
		II	Diffuse Fibrillary	8	16%
		III	Anaplastic	3	6%
		IV	Glioblastoma Multiforms	19	38%
Ependymal tumors	5	I	Myxo-papillary ependymoma	1	2%
		II	Ependymoma	3	6%
		III	Anaplastic ependymoma	1	2%
Oligo-glial tumors	1	III	Anaplastic oligodendrglioma	1	2%
Mixed glioma	1	III	Anaplastiv astro-oligodendroglioma	1	2%
Total	50	-	-	50	100%

Table 2: WT1 scores, Mean MVD and Mean mitosis among different grades of glial tumors

				WT score							
Tumor grade	Total	Mean mitosis	MVD	0	1	2	3	4	5	6	Mean WT score
Grade I	14 (28%)	0.0	69.7±36.6	7	0	3	1	2	1	0	1.5±1.8
Grade II	11 (22%)	0.36±0.5	72.9±27.4	2	0	4	1	4	0	0	2.4±1.5
Grade III	6 (12%)	2.5±1	115.5±66	0	0	2	0	1	2	1	4±1.6
Grade IV	19 (38%)	2.57±1.3	139.3±57.8	0	0	1	1	3	6	8	5±1.1
Total	50 (100%)	-	-	9 (18%)	0 (0%)	10 (20%)	3(6%)	10(20%)	9(18%)	9(18%)	-
P-value	-	< 0.05*	< 0.05*				--				< 0.05*

* Significant differences among different tumor grades (MANOVA).

Table 3: Mean age of patients and necrosis of grade IV tumors among all WT1 scores

WT1 score	Mean age	Mean GBM Necrosis%
0	21.3±12	-
1	-	-
2	22.5±19	5%
3	36±11	10%
4	32.3±24	Mean 6.3±3.2%
5	46.6±18	Mean 41.6±29.7%
6	46.5±40	Mean 30.6±27%
P-value	> 0.05 (NS)	> 0.05 (NS)

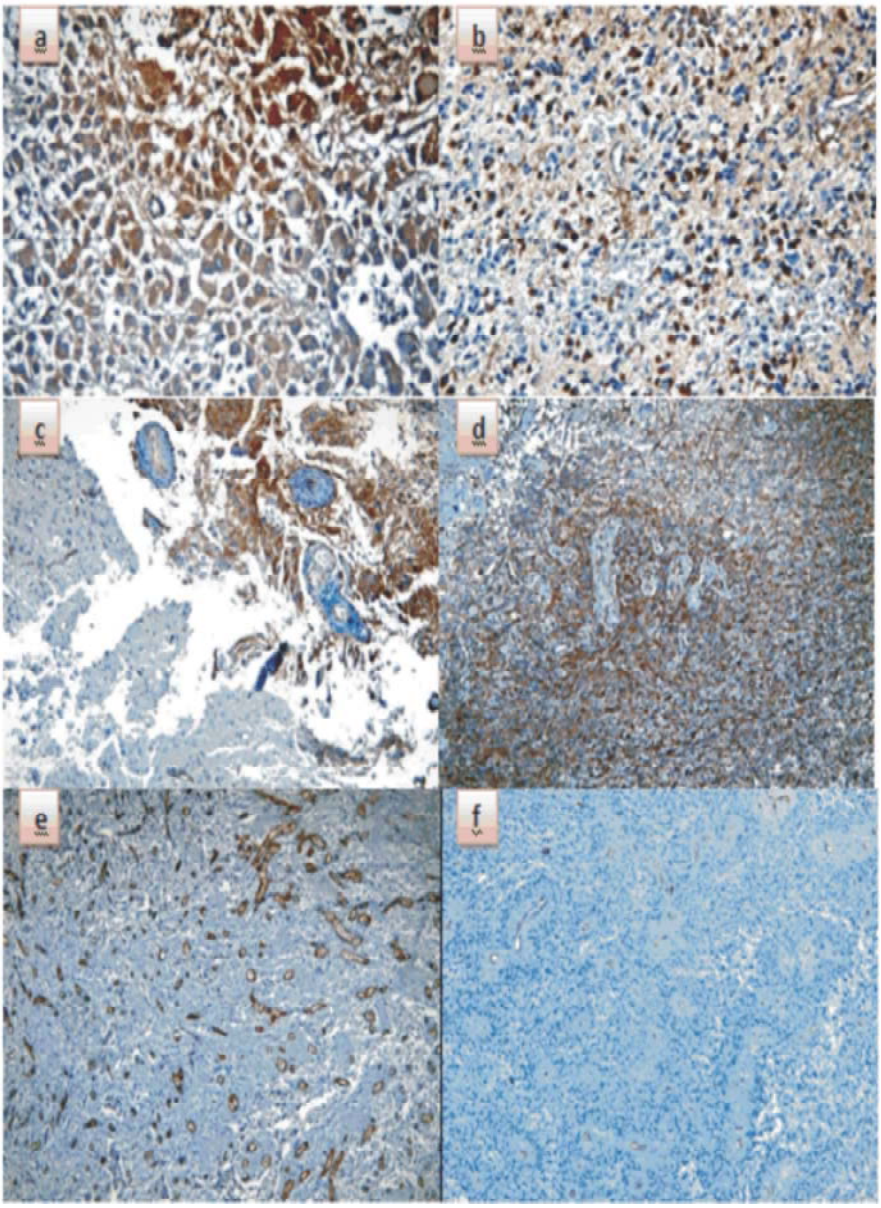

Fig. 1: (a, b, c, d): Positive expression for WT1 in glial tumors; (a) SEGA, score 5 (b): Diffuse fibrillary astrocytoma, score 4 (c, d); GBM with score 6, adjacent normal brain in (c) showed negative expression with positivity in vascular endothelium. **(d)** High vascularity in GBM as revealed by CD 34 expression. **(f)** Negative expression for WT1 in ependymona with positivity in vascular endothelium.

correlation. However there was strong negative; inverse; relation between MVD and WT1 score 0 (rho value -0.940) with p-value close to significance (=0.51) and regarding other scores, rho values showed negative correlation with scores 1 up to 4 and positive correlations with scores 5-6, but as well statistically non-significant. The same finding was detected as regards correlation between WT1 score and mitotic activity where strong positive correlation (rho=949) was found between mitotic activity and WT1 score 6 and strong negative correlation (rho= -949) with WT1 score 0 were detected, but p-value was 0.51 for both, which is again close to significance.

The percentage of tumoral necrosis among the 19 cases of glioblastoma multiforms was estimated subjectively in each slide, it ranged from 5% up to 85%. The mean percent necrosis was calculated from each WT score; from 4 to 6; where most cases clustered (17) and then statistical analysis using MANOVA; revealed non-significant differences between means among different WT1 scores, with p-value >0.05. The means age of patients for each WT1 score; was calculated and MANOVA, revealed again non-significant differences between age means among different scores, with p-value >0.05.

DISCUSSION

The current study evaluated WT1 expression in 50 glial tumors, as a possible prognostic and therapeutic marker like HER-2 in breast. We started by estimating the percentage of tumoral expression of WT1 which was 86% (43/50) of total cases ranging from score 2 to 6. This was close to Mahzouni and Meghdadi [33], who found that 71 of 73 (97.3%) studied primary astrocytic tumor samples expressed WT1 protein in the cytoplasm and agrees with Oji *et al*. [15], who found that WT1 protein was overexpressed in almost all of the astrocytic tumors (5/6 of low grade tumors and all the 18 high grade ones). The current study revealed significant differences between mean WT1 scores between different tumor grades, emphasizing the fact that WT1 score increases significantly with increasing tumor grade. This is in agreement with those reported by Oji *et al*. [15], who found that a high expression level of WT1 protein correlated with a high grade of astrocytic tumors. Hashiba *et al*. [27] as well found higher expression scores in higher grade glial tumors suggesting that WT1 gene may be involved in proliferation and malignant transformation in various glial tumors. Schittenhelm *et al*. [34] found that WT1 was expressed in 52% of all diffuse astrocytomas, but tumors with more than 75% positive WT1 cells should prompt the diagnosis of a high grade glioma. Mahzouni and Meghdadi [33] also reported that mean rank of WT1 index was significantly different between various grades of astrocytic brain tumor. So we concluded that WT1 can be considered as an oncogene involved in glial tumerogenesis and that its overexpression definitely is associated with higher tumor grade and thus prognosis.

The cytoplasmic localization of WT1 expression in the current study is in harmony with Oji *et al*. [15], who detected WT1 protein in the cytoplasm of astrocytic tumor cells with positive WT1staining, which suggested possible functions of WT1 other than as a transcription factor. Niksic *et al*. [35] suggested involvement of WT1 in RNA metabolism. Maximum immunostaining in all positive cases were seen in highly cellular portions with high vascularity which agrees with Hashiba *et al*. [27], who found that WT1 protein was strongly expressed in the anaplastic portions and areas with perivascular proliferation and high cellularity, implying that WT1 gene might be important in glial tumor cell proliferation. MVD for each tumor grade revealed significant differences between different tumor grades, emphasizing the fact that MVD increases significantly with increasing tumor grade and thus serves well as is a marker of prognosis. Cavalcante *et al.* [36] concluded that greater MVD would be obtained with increasing tumor grade. Such a relationship was found between the LGAs and AAs, but not for the GBMs, however *Yao et al*. [37] found a significant difference between GBMs and LGAs, while Leon *et al*. [38] considering only AA and GBM cases, showed that there was a significant association between microvascular grade and histology.

Statistical analysis revealed non-significant correlation between WT1 score and MVD. However; the fact that there were a strong negative inverse relation between higher MVD and WT1 negative expression (rho value -0.940) with p-value close to significance (=0.51) and a positive relation with high WT1 expression (scores 5-6), but yet statistically non-significant; warrants further evaluation with larger number of cases to confirm or discard the possible role of WT1 in glial tumors angiogenesis. Wagner *et al*. [30] concluded that WT1 increased the proliferation and migration of endothelial cells by regulating the levels of the Ets-1 transcription factor, but he studied 113 solid tumors of different types and organs**.** McCarty *et al.* [26] found a correlation between levels of WT1 expression and VEGF expression in Ewing sarcoma cell lines and that WT1 plays a key role in optimizing the response of tumor cells to hypoxia.

The relationship between WT1 expression and angiogenesis in glial tumor was not thoroughly evaluated by other studies. Our study showed that there is a possible but not definite role of WT1 in glial tumor angiogenesis and thus this role and the possible target genes involved in this process need repetitive evaluations and investigations. Statistical analysis revealed significant differences between means of mitotic activity among different tumor grades, emphasizing the fact that mitotic activity increases with increasing tumor grade. Though we relied on routine sections and not proliferative index with MIB-1, but our result agrees with Mahzouni and Meghdadi [33] who found that along with the progression of tumor grade, MIB-1 staining index demonstrates a continuous increase, which verifies higher proliferative activity in high-grade tumors. Previous studies support this finding where MIB-1 is reported to be correlated with tumor grade as well as with mitotic activity [27-39, 40]. Thus mitotic activity serves well as a marker of prognosis.

As regards correlation between WT1 score and mitotic activity, again strong positive correlation (rho=949) was found between mitotic activity and WT1 score 6 and strong negative correlation (rho= -949) with WT1 score 0 were detected, but p-value was 0.51 for both, which is again close to significance and thus requires repeated evaluations. Clark *et al.* [28] and Chen *et al*. [41] found that aberrant expression of WT1 in glioma cells contributes to enhanced cell proliferation in vitro and in vivo and resistance to radiation- and chemotherapy. Mahzouni and Meghdadi [33] found significantly positive correlation was between WT1 protein expression and MIB-1 staining index implying that WT1 protein expression might be related to both tumor cell proliferation and tumor grade. Our study revealed non-significant correlation between WT1 score and extent of necrosis in grade IV tumors (GBM) and revealed non-significant correlation between mean ages of patients among all WT1 score groups. These points have not been properly evaluated till now in other studies and thus needs further investigations.

In conclusion WT1 is expressed in a considerable percent of glial tumors and bears relation to tumor grade and thus prognosis and bears strongly a possible but yet not well established correlation with tumor angiogenesis and proliferative activity. This makes it a useful marker for prognosis and possible target for cancer therapy. However to proceed with this we must carry out further studies to confirm such role, declare the mechanism, which is probably related to downstream target genes. The scoring of WT1 expression is also not standardized between researches and we recommend it to be either negative (score 0), weak (scores 1-2), moderate (scores 3-4), or strong (scores 5-6) and consider positivity from scores 3 to 6 (moderate or strong staining) and negativity from scores 0 to2 (absent or weak staining).

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