WT1 in astrocytomas: Comprehensive evaluation of immunohistochemical expression and its potential utility in different histological grades

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Abstract
BACKGROUND: Wilms’ tumor 1 (WT1) mutation has recently been detected in gliomas. Growing data indicate that WT1 mutation plays a causal role in gliomagenesis and is overexpressed in most glioblastomas. An emerging immunotherapy targeting WT1 has shown to be effective in resistant glioblastomas in clinical trials. WT1 expression and its potential utility in various grades of astrocytomas is still unclear and needs further elucidation. The evaluation of WT1 can be done by molecular or immunohistochemical methods. As immunohistochemistry is easier with wider routine use, immunooexpression of this biomarker was studied.
AIM: The aim of this study was to characterize WT1 immunoexpression across different histological grades of astrocytomas to routinely aid in diagnosis and reproducibility and to assess the association between WT1 and immunomarker isocitrate dehydrogenase (IDH1).
MATERIAL AND METHODS: This was an observational prospective study on 79 cases of astrocytomas.
RESULTS: Seventy-nine astrocytomas including 11 recurrent tumors were assessed for WT1 by immunohistochemistry. WT1 expression was detected in all astrocytomas (100%). The control group of reactive gliosis was negative. WT1 score correlated with histological tumor grades (P < 0.001) with higher score in higher grade. It was also observed that different tumor grades depicted two distinct expression patterns. WT1 score and pattern were valuable in differentiating high- and low-grade astrocytomas.
CONCLUSION: This study supports the oncogenic role of WT1 in astrocytomas. WT1 was found to be valuable in distinguishing different grades of astrocytomas. WT1 can aid in differentiating neoplastic process from reactive gliosis, particularly in recurrent tumors. Higher expression in glioblastomas supports its immunotherapeutic potential.

Key Words: Astrocytomas, gliomas, immunohistochemistry, WT1

Introduction
Wilms’ tumor 1 (WT1) encodes for a transcriptional factor which plays an important role in promoting growth and differentiation of cells.[1] Its role has been implicated in various malignancies. WT1 was reported first in Wilms’ tumor.[2] Subsequently, it was found to be overexpressed in leukemias and various solid tumors including breast and ovarian malignancies.[3] Recent studies have shown its role in gliomas.[3-8] Overexpression of WT1 has been found in high-grade astrocytomas[5,7,9] and in varying frequencies over different histological grades.[4,5] However, its utility has not been sufficiently substantiated and controversies still exist regarding its potential. Exquisite molecular techniques to analyze WT1 expression have been established. Immunohistochemical approach for WT1 is a useful and less-labor-intensive method to detect WT1 mutations. Some studies have shown WT1 mRNA levels compared with the immunohistochemical score presenting similar results between immunohistochemistry (IHC) and molecular data.[5] However, the immunohistocharacteristics of WT1 over various grades need further investigation. Lately, clinical trials of cancer immunotherapy targeting WT1 protein have shown promising results in glioblastomas chiefly in resistant cases, suggesting that WT1 can be a possible target for immunotherapy in high-grade gliomas.[10] The presence of WT1 mutation needs to be tested before therapy and can facilitate clinical decisions. Elucidation of the complete role of WT1 in astrocytomas may help in the diagnosis and perhaps open new avenues for a possible target for cancer therapies in astrocytomas.[3,6] Therefore, we studied WT1 by IHC in astrocytomas to evaluate its expression in different grades of astrocytomas. In addition, expression in reactive gliosis was also studied. This is the first study in Indian population to the best of our knowledge.

Materials and Methods
This study included 79 prospective cases of astrocytomas received in the department of histopathology over a 24-month period between January 2015 and December 2016. Astrocytomas with significant oligodendroglial component or with insufficient material for IHC were excluded. In addition, 20 cases of known reactive gliosis formed the control group. The study was approved by the Medical Ethics Committee of the institution. The clinical data including patient age, sex, and tumor site were recorded.

Histopathological examination
The surgical specimens were fixed in 10% buffered neutral formalin and processed for paraffin embedding. For histopathological examination, 3-μm-thick sections were stained with hematoxylin and eosin. Histological diagnosis and grading were done according to the World Health Organization (WHO) classification of central nervous system tumors[11,12] based on cellularity, pleomorphism, number of mitosis per 10 high-power field (HPF), presence or absence of microvascular proliferation, and necrosis.

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**Immunohistochemistry**

For IHC analysis, additional sections were prepared for conventional IHC with WT1 antibody (GF-H2, Cell marque, prediluted), Ki-67 (MIB-1, Dako, prediluted), and isocitrate dehydrogenase (IDH1) (IDH1-R132H monoclonal antibody) according to the standard procedures on automated immunostainer BioGenex X Matrix (49026 Milmont Drive, Fremont, CA 94538, USA).

**Immunohistochemical assessment**

All cases were studied for histomorphology and IHC by two experienced pathologists (SJ and AM) independently. WT1 was evaluated for proportion score and pattern of staining. Cytoplasmic staining of neoplastic cells showing moderate-to-high intensity was considered positive. Weak or equivocal staining was excluded. WT1 expression was evaluated using a semiquantitative scoring method and scored as 0 (no staining), 1 (singular positive cells, ≤1%), 2 (>1–25%), 3 (26-50%), and 4 (>50%), which was comparable to that proposed earlier by Rausher et al.\(^4\) For counting the immunopositive cells, 10 high-power (40×) fields were selected and systematically randomized throughout the section. Ki-67 expression was calculated as percentage of cells with nuclear positivity. The correlation of WT1 expression and histological grade was calculated.

**Statistical analysis**

Descriptive statistics was analyzed with SPSS version 17.0 software. Continuous variables are presented as mean (min–max). Categorical variables are expressed as frequencies and percentages. The Pearson Chi-square test or the Chi-square test of association was used to determine if there is a relationship between two categorical variables. Probability (\(P\)) values <0.05 were considered statistically significant.

**Results**

The spectrum and demographic details of astrocytomas are shown in Table 1.

**Characteristics of patients**

There was a male predominance (male: 75.95%; females: 24.05%). The mean age was 41.86 years with age range of 3–77 years. The most common location was frontal lobe followed by temporal, parietal, and insular region. Grade IV was the most common (48.10%) followed by grade II (34.18%).

**Table 1: Summary of demographic details and spectrum of astrocytomas**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No. of patients (n, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>79</td>
</tr>
<tr>
<td>Mean age</td>
<td>41.86 years</td>
</tr>
<tr>
<td>Male</td>
<td>60 (75.95%)</td>
</tr>
<tr>
<td>Female</td>
<td>19 (24.05%)</td>
</tr>
<tr>
<td>Pathological grade</td>
<td></td>
</tr>
<tr>
<td>Astrocytoma I</td>
<td>9 (11.40%)</td>
</tr>
<tr>
<td>Astrocytoma II</td>
<td>27 (34.18%)</td>
</tr>
<tr>
<td>Astrocytoma III</td>
<td>5 (6.32%)</td>
</tr>
<tr>
<td>Astrocytoma IV</td>
<td>38 (48.10%)</td>
</tr>
<tr>
<td>Recurrent tumors</td>
<td>11 (13.92%)</td>
</tr>
</tbody>
</table>

**WT1 immunohistochemical expression**

WT1 positivity was detected in all astrocytomas (79/79; 100%). Positivity was detected in cytoplasm, astrocytic processes, and fibrillary tumor matrix. Immunostaining in the endothelial cells served as positive internal control for WT1. WT1 was positive in neoplastic areas, whereas adjacent normal glial tissues were negative [Figure 1a and b]. All cases of reactive gliosis in the control group were negative for WT1.

Table 2 summarizes the score and pattern of WT1 expression in different grades of astrocytomas. The immunostaining proportion score varied from 2 to 4. None of the cases showed a score of 1. Higher expression was found in more cellular areas. In tumors showing heterogeneity, WT1 score was higher in high-grade areas compared to low-grade areas. Staining was absent in necrotic areas. High WT1 expression of score 4 was seen in a majority of glioblastomas (31/38; 81.6%) [Figure 2a and b] and anaplastic astrocytomas (5/5; 100%) [Figure 2c and d]. In contrast, score 4 was seen in 2/27 (7.4%) grade II and 2/9 (22.2%) grade I astrocytomas. Majority of the grade II tumors showed score 3 [Figure 2e and f]. Score 2 was observed only in grade I (3/9; 33.3%) and grade II (1/27; 3.7%) astrocytomas. A statistically significant association of WT1 score and tumor grade was observed (\(P < 0.001\)) with higher score in higher grade of tumor. Combining grade III and IV as high-grade astrocytomas and grade II and I as low-grade astrocytomas, WT1 expression score of 4 was found in 83.7% of high-grade astrocytomas and in 11.4% of low-grade astrocytomas and was found statistically significant in differentiating these two categories (\(P\)-value <0.001).

WT1 immunostaining pattern varied from dense to loose in different histological tumor grades [Figure 2b, d and e]. In the dense pattern, astrocytic processes were strongly and diffusely stained with contiguous areas. In the loose pattern, the delicate fibrillary processes showed patchy staining without contiguous areas. A dense pattern was seen in 100% of high-grade astrocytomas (43/43) with a predominant dense pattern in 74.4% cases (78.9%; 30/38 of grade IV astrocytomas and 40%; 2/5 of grade III astrocytomas), whereas at least a focal dense pattern was observed in the remaining (25.6%) high-grade astrocytomas.
astrocytomas. The latter cases also showed a high score of 4. In contrast, 96% (26/27) of grade II astrocytomas showed a predominant loose pattern. Only one case of grade II astrocytomas showed a focal dense pattern. All the pilocytic astrocytomas showed a classical biphasic pattern of loose and dense areas [Figure 3a and b]. Dense pattern was statistically significant in differentiating high grade from grade II astrocytomas (P < 0.001). *WT1* expression score and pattern revealed a close association with each other wherein tumors with high *WT1* score 4 showed a dense cytoplasmic pattern in 75% cases and low scores had a loose cytoplasmic pattern in 77.1% cases.

**Correlation with Ki-67 index**
A significant correlation was observed between higher WT1 score and higher Ki-67 proliferation index. Ki-67 index of >5% had a higher WT1 score of 4 in 75% cases and was statistically significant (P-value <0.001).

**WT1 in recurrent astrocytomas**
Positive *WT1* was seen in all recurrent tumors (11/11). Among recurrent tumors, 5 of 11 cases, no histomorphological therapy-related changes were observed. In these cases, *WT1* expression was not affected by therapy and expression was similar to the de-novo cases within the individual grades. Six of 11 cases showed therapy-related changes like radiation necrosis and reactive astrocytes. In these cases, score evaluation was difficult; however, detectable *WT1* expression was present.

IDH1 immunostaining findings are depicted in Table 3. In grade IV astrocytomas, most cases were IDH1 negative (31/38; 81.6%) and only seven cases of glioblastoma (7; 18.4%) showed IDH1 positivity, which included 6/7 secondary (recurrent) and 1/3 primary glioblastoma. These IDH1-positive glioblastomas also showed a high WT1 score of 4. The frequency of IDH1 positivity in grade II astrocytomas was high (21/27; 77.7%); in contrast, the WT1 score was lower in this grade. An inverse relationship was observed between WT1 scores and IDH1 positivity. There was a negative correlation between WT1 scores and IDH1 positivity.

**Discussion**
Astrocytomas are the most common gliomas constituting more than 60% of all brain tumors.\[12\] *WT1* is a recently introduced molecular marker in gliomas found to play a role in gliomagenesis.\[4,6,7\] The *WT1* was first identified as a tumor suppressor gene arising due to frequent deletions at chromosome 11p13 region involved in the development of a childhood renal neoplasm, Wilms’ tumor.\[14\] *WT1* gene encodes a zinc finger transcriptional factor involved in cell growth and differentiation. The oncogenic role of *WT1* has been recognized in several hematological malignancies, melanomas, and other solid tumors in recent times.\[3\] In gliomas, few studies have shown that *WT1* expression is a common finding and have suggested its role in the tumorigenesis of malignant astrocytic tumors.\[7,8\] The occurrence of *WT1* in high proportion

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**Table 2: Correlation of percentage positivity (score) and patterns of *WT1* with different histological grades in astrocytomas**

<table>
<thead>
<tr>
<th>Tumor grade</th>
<th>WT1 score*</th>
<th>WT1 pattern**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Score 1</td>
<td>Score 2</td>
</tr>
<tr>
<td>Grade I (9)</td>
<td>0</td>
<td>3 (33.3%)</td>
</tr>
<tr>
<td>Grade II (27)</td>
<td>0</td>
<td>1 (3.7%)</td>
</tr>
<tr>
<td>Grade III (5)</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>Grade IV (38)</td>
<td>0</td>
<td>0%</td>
</tr>
</tbody>
</table>

*WT1 score and **WT1 pattern showed significant correlation (P<0.001) with histological tumor grade
Table 3: Immunohistochemical findings of IDH1 and WT1 scores in various grades of astrocytomas

<table>
<thead>
<tr>
<th>Tumor grade (cases)</th>
<th>IDH1 Positive (n)</th>
<th>IDH1 Negative (n)</th>
<th>WT1 Score 2</th>
<th>WT1 Score 3</th>
<th>WT1 Score 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade I (9)</td>
<td>0</td>
<td>9</td>
<td>3 (33.3%)</td>
<td>4 (44.4%)</td>
<td>2 (22.2%)</td>
</tr>
<tr>
<td>Grade II (27)</td>
<td>21</td>
<td>6</td>
<td>1 (3.7%)</td>
<td>24 (88.9%)</td>
<td>2 (7.4%)</td>
</tr>
<tr>
<td>Grade III (5)</td>
<td>3</td>
<td>2</td>
<td>0%</td>
<td>0%</td>
<td>5 (100.0%)</td>
</tr>
<tr>
<td>Grade IV (38)</td>
<td>7</td>
<td>31</td>
<td>0%</td>
<td>7 (18.4%)</td>
<td>31 (81.6%)</td>
</tr>
</tbody>
</table>

of gliomas while its absence in normal astrocytes suggests that WT1 has an oncogenic role and is a valuable marker for gliomas.[5] Furthermore, clinical trials of targeted immunotherapy against WT1 protein have shown promising results in glioblastomas. Studies have revealed that transient WT1 silencing increases the sensitivity of tumors to radiotherapy and chemotherapy,[6] suggesting that WT1 can be a probable target for immunotherapy in aggressive high-grade gliomas. Evaluation of WT1 is therefore important for neuropathologists and oncologists to predict the response to anti-WT1 therapy. The immunohistochemical method to assess WT1 mutation appears to be more suitable for routine use. Utility and validation of WT1 immunoexpression have not been sufficiently substantiated and controversies still exist on its potential. The immunohistochemical approach and potential role of WT1 in astrocytomas, therefore, need further elucidation. The aim of this study was to further specify WT1 expression across different histological grades of astrocytomas to increase diagnostic clarity and reproducibility in routine diagnostic work.

In the present study, WT1 expression was found in all the 79 cases of astrocytomas (100%). Previous studies have shown similar results with positivity varying between 80.9% and 100%. [3-6] Although most molecular studies of WT1 have shown 100% positivity,[5,6] some studies using IHC approach have found absent expression in a small percentage of astrocytomas. Mahzouni et al. in his study of 73 astrocytomas found WT1 protein expression in most cases (97.3%) with only two cases, one of glioblastoma and one pilocytic astrocytoma with no WT1 protein expression.[15] Rauscher et al., in their study, found some anaplastic astrocytomas and glioblastoma lacking WT1 expression. They ascribed this negative expression in high-grade tumors to the younger age of patients and tumors possessing IDH1 mutations.[4] In our study, none of the astrocytomas were negative for WT1, although the percentage of expression varied. In contrast, all the 20 cases of reactive glial tissue in the control group were negative for WT1, thus supporting the oncogenic role of WT1 in astrocytomas. Clark et al. had also observed that WT1 protein was not detected in the normal glial cells.[3] Bourne et al., however, suggested that WT1 marker is not dependable in discriminating reactive from neoplastic astrocytes as reduced extent of WT1 expression might also be present in reactive astrocytes.[16]

We found a statistically significant correlation between WT1 expression and WHO histological tumor grade in astrocytomas. Higher expression of WT1 was seen in higher tumor grade supporting its role in tumorogenesis and tumor progression. Majority of glioblastomas (81.6%) and anaplastic astrocytomas (100%) showed score 4 WT1 staining positivity. It can, therefore, be suggested that higher expression favors a higher grade of tumor and can be useful in differentiating high and low-grade astrocytomas when dealing with challenging biopsies. Hashiba et al. observed that astrocytomas showed strong expression of WT1 protein in high-grade astrocytomas, especially in glioblastoma with WT1 expression score of 3 or 4 in 31 of 34 cases (91.2%) and the expression decreased in frequency in the lower grades.[8] Consistent with our results, Mahzouni et al. found that high WT1 expression was significantly more in glioblastoma (87.5%) as compared to low-grade astrocytomas.[12] Clark et al. suggested that WT1 aids to maintain the high proliferative rate in glioblastoma. They found that downregulation of WT1 caused reduced tumorigenicity of the in-vitro as well as in-vivo glioblastoma cell line; hence, WT1 can serve as a promising target for new molecular glioblastoma therapies.[3] In the present study, two cases of pilocytic astrocytomas also showed score 4. Similar observation was made earlier.[4] In a study of 829 gliomas, Rauscher et al. also observed that WT1 scores significantly vary between astrocytic and oligodendrogial tumors and suggested that WT1 expression might contribute in distinguishing astrocytomas from oligodendrogliomas.[4] This facet was, however, not explored in the present study.

In the literature, WT1 expression in astrocytomas is described as cytoplasmic staining in the delicate cell processes as opposed to the nuclear immunostaining in Wilms’ tumors. This is elucidated by the ability of WT1 protein to transport from the nucleus to the cytoplasm.[17] Although WT1 immunoexpression in gliomas has been studied, the pattern of WT1 protein expression has not been described. Hashiba et al. had observed WT1 as speckled and heterogeneous pattern in many cases contrasting to a homogenous appearance in a few of them, but its significance was not further highlighted.[8] In this study, we observed two types of WT1 patterns of dense and loose types in different grades of astrocytomas. Dense pattern diffusely or focally was seen in a majority of high-grade astrocytomas suggesting that dense pattern is indicative of high-grade astrocytomas. Loose pattern was frequently encountered in low-grade astrocytomas. The different staining patterns of WT1 may help in challenging the distinction of these entities. Some of the pilocytic astrocytomas (4/9) also showed focal dense pattern with biphasic loose and dense areas. The most plausible explanation for this phenomenon can be that biphasic pattern is reminiscent of the biphasic histology characteristic of pilocytic astrocytomas. In pilocytic astrocytomas, one needs to be cautious as it can show both focal high expression score and dense pattern. Interpretation should be done along with characteristic histomorphology findings and Ki-67 index in pilocytic astrocytomas. We also observed a statistically significant correlation of WT1 score and pattern (P < 0.001). Based on our results, it is suggested that interpretation of WT1 should be based both on proportion scoring as well as on the pattern of staining.
High score and dense pattern are indicative of a high-grade astrocytoma and vice versa. The probable explanation for the characteristic staining pattern of loose or dense patterns is that they are likely to have arisen due to the cell density of WT1 expressing glioma cells a which commonly relates to the WHO grade of glioma. Proliferative activity of astrocytomas by monoclonal antibody Ki-67/MIB-1 is an established method to assess tumor biology and prognosis. In this study, positive correlation of WT1 and Ki-67 was seen with higher WT1 expression showing high Ki-67. Mahzouni et al. and Hashiba et al. had also observed a significant positive correlation between WT1 expression and MIB-1 staining index, suggesting that WT1 protein expression might be associated with tumor cell proliferation and tumor grade.

WT1 was found useful in differentiating reactive gliosis from tumor recurrence in cases showing treatment-related changes like necrosis and reactive gliosis. Our data show that detectable expression of WT1 is useful in differentiating recurrent tumors from reactive gliosis. A study by Schittenhelm et al. showed similar WT1 expression in primary and secondary astrocytic tumors. Rauser et al. observed that WT1 positive cases were significantly fewer in recurrent tumors. However, they have not specified treatment-related changes.

Similar to our study, IDH1 immunopositivity has been observed previously with higher frequency in grade II and secondary grade IV astrocytomas. Rauscher et al., in their study found IDH1 mutation to be associated with less WT1 positive cases as compared to those having IDH1 wild type. Although we observed an inverse relationship between WT1 scores and IDH1 positivity, there was no statistically significant correlation.

**Conclusion**

Our results support the oncogenic role of WT1 in astrocytomas. We have attempted to further specify the WT1 expression in different histological grades of astrocytomas. WT1 expression significantly correlated with WHO histological tumor grade and Ki-67 index. It is suggested that interpretation should be based on both score and pattern. Higher expression score and dense pattern favor higher tumor grade, whereas lower expression and loose pattern is suggestive of lower tumor grade. The scoring and distinctive patterns of WT1 can aid in distinguishing high-grade and low-grade astrocytomas. However, one needs to be careful in pilocytic astrocytomas in which there is a mixed expression. It was found that WT1 can also help in differentiating tumor recurrence from reactive gliosis. WT1, therefore, appears to be an attractive IHC marker to be used concomitantly with other IHC markers in astrocytomas. In addition, the frequent expression of WT1 in astrocytomas supports its promising role in immunotherapy and its potential to guide patient selection for targeted immunotherapy. This study, however, lacks correlation with mRNA expression of WT1 due to limited resources. Further studies with a larger sample size and comparison with molecular mRNA expression of WT1 are required to explore the full potential of WT1 in diagnosis and therapy.

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Nil.

**Conflicts of interest**

There are no conflicts of interest.

**References**