Sensitivity Assessment of Wilms tumor gene (*WT1*) expression in Glioblastoma Using qPCR and Immunohistochemistry and its association with *IDH1* mutation, and recurrence interval

Maheer Kurdi (✉ Ahkurdi@kau.edu.sa)

King Abdulaziz University <https://orcid.org/0000-0002-8979-3849>

Nadeem Shafique Butt

King Abdulaziz University

Saleh Baeesa

King Abdulaziz University

Abudukadeer Kuerban

King Abdulaziz University

Yazid Maghrabi

King Faisal Specialist Hospital and Research Centre - Jeddah

Anas Bardeesi

King Faisal Specialist Hospital and Research Center

Rothaina Saeedi

King Abdulaziz University

Badrah Alghamdi

King Abdulaziz University

Ahmed I. Lary

King Abdulaziz Medical City - Jeddah

Fawaz Mohammed

King Abdulaziz University

Research

Keywords: Glioblastoma, IDH1 mutation, WT-1 Expression, Chemotherapies, PCR sensitivity

Posted Date: March 10th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-290379/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published at *Biologics: Targets and Therapy* on July 16th, 2021. See the published version at <https://doi.org/10.2147/BTT.S323358>.

Abstract

Background

The Wilms tumor 1 (*WT1*) gene has recently been shown to play a role in gliomagenesis, making it a potential immunotherapy target in glioblastomas. We aimed to investigate the most sensitive method to detect *WT1* expression in glioblastoma and explore relationship between *WT1* expression and isocitrate dehydrogenase-1 (*IDH1*) mutation.

Methods

Clinical and biological data were collected from 44 patients with totally resected glioblastomas, treated with adjuvant therapies, in the period between 2015 and 2019. *WT1* protein expression was assessed in all cases using IHC while its gene expression was assessed in 13 clustered samples using quantitative polymerase chain reaction (qPCR). *IDH1* mutation was assessed using immunohistochemistry (IHC). McNemar test was used to compare the sensitivity between IHC and RT-qPCR for *WT1* gene expression detection. Kaplan Meier curves were used to compare the distribution of recurrence-free interval (RFI) between *IDH1* and *WT1* expression groups.

Results

The mean age was 54-years, with a male to female ratio 1.45. *IDH1*-wildtype was found in 26 cases (59.1%) and the remaining 18 cases (40.9%) were *IDH1*-mutant. Through IHC, *WT1* was overexpressed in 32 cases (72.7%), partially expressed in 9 cases (20.5%) and not expressed in only three cases (6.8%). For the 13 cases tested by qPCR, 6 cases showed *WT1* gene up-regulation and 7 cases showed *WT1* down-regulation. There was no significant difference in *WT1* expression among cases with different RNA concentrations regardless the testing method (P -value < 0.05). However, the difference between IHC and qPCR revealed 83% sensitivity, 28.5% specificity and 53% accuracy. *IDH1*-mutant cases with *WT1* overexpression showed significant difference in recurrence interval (P -value < 0.048). This significance was not seen among *IDH1*-mutant cases with *WT1* partial or no expression (P -value = 0.56). There was also no significant difference in recurrence interval among *IDH1*-wildtype cases with *WT1* partial or overexpression (P -value = 0.83).

Conclusions

Parallel testing for *WT1* expression using IHC and qPCR is not reliable. However, IHC provides more accurate results than does qPCR, which runs on fragmented tissue with indeterminate RNA concentrations. Moreover, *IDH1*-mutant glioblastomas with *WT1* overexpression are associated with slow recurrence interval particularly if temozolomide with additional chemotherapies are used.

Introduction

The Wilms tumor 1 (*WT1*) gene encodes a zinc finger transcriptional factor that plays an important role in cell growth and differentiation (1). *WT1* been implicated in various malignancies. *WT1* was first identified as a tumor suppressor because of frequent chromosome 11p13 region deletions observed in childhood renal neoplasm and Wilms tumor (2-3) and was then found to be overexpressed in leukemias and various solid tumors including breast and ovarian malignancies (4). Recent studies have shown that *WT1* has a role in gliomagenesis (5). Consistently, *WT1* overexpression has been found in high-grade gliomas (4) (6-7). However, the potential of using *WT1* expression as a molecular marker has not been sufficiently substantiated. Recently, clinical trials of cancer immunotherapy targeting WT1 protein have shown promising results in glioblastomas, particularly in resistant cases. These results suggest that WT1 is a possible target for immunotherapy in high-grade gliomas (8) and also could increase the sensitivity of glioblastoma to chemoradiotherapy. The immunohistochemical approach is considered the standard method to detect WT1 protein expression. However, some studies have shown that *WT1* mRNA levels are presenting similar results to that of the immunohistochemical score (4). Therefore, the most accurate method for testing *WT1* expression in glioblastoma is not obviously clear. This study was designed to investigate whether IHC or qPCR are more for detecting *WT1* gene expression in glioblastoma cases.

Rauscher et al found that some high-grade gliomas lacked *WT1* expression while Manocha et al identified an inverse relationship between *WT1* scores and *IDH1* mutation (9). They ascribed this negative expression in high-grade tumors to the younger age of patients and tumors possessing *IDH1* mutations (10). Therefore, this study was also designed to explore the relationship between *WT1* expression and *IDH1* mutation and how this influences patient outcome.

Material And Methods

1. Sample Stratification

We included 44 patients with totally resected glioblastoma, who received adjuvant therapies, from one medical institution in Saudi Arabia between 2015 and 2019. Ethical approval for this study was granted by the National Biomedical Ethics Committee at King Abdulaziz University (HA-02-J-008) (Reference No. 189-19). Histological diagnoses were made according to the World Health Organization (WHO) classification. Clinical data including age at diagnosis, gender, post-operative adjuvant therapies, type of chemotherapies, and recurrence interval, were retrieved from hospital records. Patients were stratified based on *IDH1* mutation and *WT1* expression (**Figure 1**). Standard radiotherapy of a total dose of 60 Gy and temozolomide (TMZ) (150–200 mg/m² for 6–12 cycles) were administered to all patients at the time of management. Some patients received additional chemotherapies including etoposide, bevacizumab, irinotecan, and lomustine.

2. Immunohistochemistry protocol used for *IDH1* mutation assessment

Anti-IDH1 antibody is intended for laboratory use to qualitatively identify IDH1 mutation in formalin fixed paraffin embedded tissue (FFPE) sections using an automated slide stainer. The IHC assay using anti-IDH1 R132H (Clone H09) mouse monoclonal antibody was performed with an OptiView detection kit on a BenchMark XT automated staining system (Ventana). The assay procedure consisted of deparaffinization with EZ Prep at 75°C, heat pre-treatment with Cell Conditioner for 68 minutes, and incubation with 1:20-1:50 diluted antibody for 32 min at 37°C. Slides were counterstained with hematoxylin II and bluing reagent for 16 minutes. Sections in which > 10% of tumor cells were positively stained were defined as mutant IDH1 (**Figure 2**).

3. Assessment of WT1 expression through IHC and RT-qPCR

a. Assessment of WT1 protein expression using IHC

Anti-Wilms tumor (WT1) antibody is intended for laboratory use to identify protein expression in FFPE sections on an automated slide stainer. The IHC assay using anti-WT1 (6F-H2) mouse monoclonal antibody was performed using the DAB detection kit from Ventana on a BenchMark XT automated staining system (Ventana). The assay procedure consisted of deparaffinization with EZ Prep at 75°C, pre-treatment with Cell Conditioner for 68 minutes, followed by incubation with 1:100–1:500 diluted antibody for 32 min at 37°C. Slides were removed from the slide stainer after counterstaining with hematoxylin II and were immersed into successive alcohol buffer for 3 min. Sections in which tumor cell cytoplasm was positively stained were defined as “WT1 expressed”. All 44 glioblastoma were stained for WT1 and the results were interpreted by a certified neuropathologist. The staining pattern was categorized as (i) overexpressed, (ii) partially expressed, and (iii) non-expressed (**Figure 3**).

b. Assessment of WT1 gene expression using reverse transcriptase-qPCR

Of the 44 enrolled glioblastoma cases, only 13 samples had adequate tissue and RNA quality for RT-PCR. This limitation should be taken into consideration during assessment of *WT1* expression in clinical practice. The samples were divided into case (n = 13) and control (n = 2) groups. IHC results showed no WT1 expression in control cases. The reference gene used for RT-PCR was glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Three replicates of threshold cycle (C_T) values for five target genes and one reference gene were used for analysis. The mean C_T and standard deviation for the reference (*GAPDH*) and target (*WT1*) genes were calculated from the RT-PCR data and analyzed by $\Delta\Delta C_T$ and ΔC_T methods. The average C_T for the control and each tested gene was calculated from the data generated by RT-PCR using the Step One System and Data Assist software. The outliers were omitted from analysis. The C_T of the target gene was normalized to the C_T of the reference gene, then the ΔC_T of the test sample was normalized to the ΔC_T of the control sample and the relative quantification (Rq) and differential expression (fold change, FC) were calculated using:

(1) ΔC_T for Ctrl or test = C_T target gene – C_T reference gene, (2) $\Delta\Delta C_T$ = ΔC_T test sample – ΔC_T control sample, (3) Relative quantification (Rq) = $2^{-\Delta\Delta C_T}$ = value*, however, Rq >1 represents up-regulation

whereas $R_q < 1$ represents down regulation of gene.

The fold change (differential expression) was also calculated. ΔC_T values for each sample were determined using: $\Delta C_T = [\text{mean } C_T \text{ reference gene} - \text{mean } C_T \text{ target gene}]$ (**Table 1**).

Statistical Methods

Data are described as frequencies and percentages. The McNemar test was used to compare the sensitivity, specificity, and accuracy of IHC and RT-qPCR for *WT1* gene expression detection. Kaplan Meier curves were used to compare the distribution of recurrence-free interval (RFI) between mutant *IDH1* and *WT1* expression groups. Recurrence interval (RI) is defined as the period after total surgical resection to the first possible date of recurrence. All statistical analyses were performed using IBM SPSS1 ver. 24 statistical software programs (SPSS Inc., Chicago, IL).

Results

Forty-four patients with resected and treated glioblastoma were included in this study. The mean patient age was 54-years, with a male to female ratio 1.45. Parietal and frontal areas were the predominant tumor location and tumors in these locations were observed in 33 cases (75%). Wildtype *IDH1* was found in 26 cases (59.1%) and the remaining 18 cases (40.9%) contained mutant *IDH1*. IHC revealed *WT1* overexpression in 32 cases (72.7%), partial expression in nine cases (20.5%), and no expression in three cases (6.8%). For the 13 cases in which *WT1* expression was tested using qPCR, six and seven cases had up- and down-regulated *WT1* expression, respectively (**Table 1-2**). Forty one patients (93.2%) received chemoradiotherapy and three patients did not receive any adjuvant therapy. Among patients who received chemotherapies, around 52% ($n = 23$) were treated with TMZ alone and 27% ($n = 17$) were treated with TMZ and additional chemotherapeutic agents. The mean recurrence time was 579 days after the total surgical resection of the tumor. Approximately 36% ($n = 16$) of patients had tumor recurrence within or after 1-year of resection while 63.6% ($n = 28$) showed recurrence before 1-year of resection. **Table 2** summarizes the descriptive distribution of the data.

1. IHC and RT-qPCR detection of *WT1* expression in patients with glioblastoma

There was no clear evidence in the literature about whether IHC or qPCR provide better results when measuring *WT1* expression. However, RT-qPCR can still be run using severely fragmented tissue but the RNA content is often barely enough to provide beneficial results.

We investigated the accuracy of both testing methods assuming that IHC, based on the previous published data, is more accurate. First, with the exception of 13 samples, most FFPE tissue from cases included in this analysis had a low RNA concentration were excluded from PCR. The remaining 13 samples had RNA concentrations > 20 nm. IHC and qPCR were used to examine and compare *WT1* expression in these 13 samples. Two cases showed downregulation (no expression) in both IHC and qPCR. A single case, with low RNA concentration (22.7 nm) showed no *WT1* expression via IHC and *WT1*

up-regulation by qPCR. Five cases that showed *WT1* overexpression on IHC had qPCR results showing downregulation. The remaining five cases had parallel results. There was no significant difference in *WT1* expression status between samples with different RNA concentrations (P -value < 0.05). The McNemar test revealed that 83% sensitivity and 28.5% specificity were achieved using IHC rather than qPCR for assessing *WT1* expression (**Table 3**). The lack of significant difference in *WT1* expression results between samples tested with IHC and qPCR indicates that both methods are not reliable to be in parallel.

2. Relationship between *IDH1* status, *WT1* expression, and recurrence interval

The recurrence interval among patients with *WT1* overexpression significantly differs between those with wildtype and mutant *IDH1* (P -value < 0.048). Cases with mutant *IDH1* showed late recurrence, after 1-year (**Figure 4**). This significance was not observed among cases with mutant *IDH1* and partially expressed or overexpressed *WT1* (P -value = 0.56). Together, these results indicate that, in cases with mutant *IDH1*, *WT1* up-regulation lowers the chance of tumor recurrence. No significant difference in recurrence interval was observed among cases with wildtype *IDH1* and *WT1* partial or overexpression (P -value = 0.83) (**Figure 4**). Moreover, patients with glioblastoma with *IDH1* mutation and *WT1* overexpression who had received TMZ and additional chemotherapies showed better survival rates and longer recurrence intervals than did those who received TMZ alone. However, no significant difference in tumor recurrence interval was observed among patients with mutant *IDH1* and *WT1* overexpression who received either TMZ alone or TMZ and additional therapies (P -value = 0.19).

Discussion

Glioblastoma is the most aggressive primary malignant brain tumor in adults. While primary and secondary glioblastomas are pathologically indistinguishable, they vary at the molecular level. After surgical resection, current standard treatment for patients with glioblastoma is combined radiotherapy and chemotherapy using either TMZ alone or TMZ with additional chemotherapeutic agents. The overall survival rate (OS) for patients with glioblastoma is around 14.6 months with a 5-year long-term survival. Nevertheless, glioblastoma remains a fatal disease and treatment strategies are palliative, and aim to improve survival rate and maintain a good quality of life.

WT1 encodes a zinc finger transcriptional factor and plays an important role in cellular growth and differentiation (1). *WT1* has been implicated in various malignancies and was first identified as a tumor suppressor gene due to frequent deletions of chromosome region 11p13 in childhood renal neoplasm (WT) (2-3). *WT1* was found to be overexpressed in leukemias and solid tumors including breast and ovarian malignancies (4). Recent studies have shown that *WT1* plays a role in gliomagenesis (5). *WT1* overexpression has been repetitively observed in high-grade gliomas (4) (6-7). However, the utility potential of *WT1* expression as a biomarker has not been sufficiently substantiated. Testing *WT1* gene or protein expression in patients with glioblastoma patients is important for planning treatment and prognostic determination. Recently, clinical trials of cancer immunotherapies targeting *WT1* have shown promising results in glioblastomas, particularly in resistant cases, suggesting that *WT1* is a potential

target for immunotherapy in high-grade gliomas (8) and increases the sensitivity of glioblastoma to chemoradiotherapy.

Although the immunohistochemical approach for assessing WT1 expression is a useful method, some studies have shown similar WT1 expression results using both IHC and molecular analyses (4). Our results showed that there is no difference in *WT1* expression results using different tests with different RNA concentrations. However, low RNA volume and concentration may give false qPCR results. This does not happen when WT1 expression being tested by IHC. Our analysis revealed that IHC has 83% sensitivity, 28.5% specificity, and 53% accuracy supporting the notion that IHC is a reliable test for WT1 expression. Moreover, our results show that parallel testing of WT1 expression using IHC and qPCR is not reliable and that IHC provides more accurate results than does qPCR.

The association between *IDH1* mutation and *WT1* expression has also not been thoroughly investigated to date. Manocha et al identified an inverse relationship between *WT1* scores and *IDH1* mutation (9). Rauscher et al found that some anaplastic astrocytomas and glioblastoma lack *WT1* expression. They ascribed this lack of WT1 expression in high-grade tumors to the younger age of patients and the presence of *IDH1* mutations in the tumors (10). In our analysis, we found that glioblastomas with mutant *IDH1* and *WT1* overexpression are associated with longer tumor recurrence interval compared with cases with wildtype *IDH1*. The association between *WT1* expression and the type of chemotherapy administered has also never been studied to date. Although *WT1* immunotherapy is currently under trial, we found that TMZ taken with additional chemotherapies may improve the survival rate and decrease the chance of tumor recurrence in patients with glioblastomas. This means that TMZ with other chemotherapies may be used as an add-on to *WT1* immunotherapy to prevent tumor regression, increase sensitivity to TMZ, and decrease tumor recurrence. Further research should be conducted in this area.

Declarations

Ethical approval: Ethical approval for this study was granted by the National Biomedical Ethics Committee at King Abdulaziz University (HA-02-J-008) (Reference No. 189-19).

Consent for publication: ready on request

Availability of the data: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing of interest: The authors declare that they have no competing interests" in this section.

Funding: The study is sponsored and funded by "Deanship of Scientific Research of King Abdulaziz University, Jeddah, Saudi Arabia" (Code No. G: 81-828-1441).

Authors contribution:

MK, idea, IRB submission, writing, study design and data, histological analysis.

NS, Statistical analysis

SB, data provider, writing, analysis

AK, study design, statistical analysis, PCR analysis, writing, editing,

YM, data entry, tissue collection, writing

AB, data entry, tissue collection

RS, data entry, tissue collection, writing

BG, data analysis, editing, submission, consultation

AL, data provider, IRB submission

FM, Tissue collection, IRB submission, IHC

Acknowledgment: n/a

References

1. Pritchard-Jones K. The Wilms tumor gene, WT1, in normal and abnormal nephrogenesis. *Pediatr Nephrol* 1999; **13**: 620–625.
2. Call KM, Glaser T, Ito CY, Buckler AJ, Pelletier J, Haber DA, et al. Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. *Cell* 1990; **60**: 509–520.
3. Nakatsuka S, Oji Y, Horiuchi T, Kanda T, Kitagawa M, Takeuchi T, et al. Immunohistochemical detection of WT1 protein in a variety of cancer cells. *Mod Pathol* 2006; **19**: 804–814.
4. Schittenhelm J, Beschoner R, Simon P, Tabatabai G, Herrmann C, Schlaszus H, et al. Diagnostic value of WT1 in neuroepithelial tumor. *Neuropathol Appl Neurobiol* 2009; **35**: 69–81.
5. Clark AJ, Ware JL, Chen MY, Graf MR, Van Meter TE, Dos Santos WG, et al. Effect of WT1 gene silencing on the tumorigenicity of human glioblastoma multiforme cells. *J Neurosurg* 2010; **112**: 18–25.
6. Kijima N, Hosen N, Kagawa N, Hashimoto N, Kinoshita M, Oji Y, et al. Wilms' tumor 1 is involved in tumorigenicity of glioblastoma by regulating cell proliferation and apoptosis. *Anticancer Res* 2014; **34**: 61–67.
7. Somasundaram A, Ardanowski N, Opalak CF, Fillmore HL, Chidambaram A, Broaddus WC. Wilms tumor 1 gene, CD97, and the emerging biogenetic profile of glioblastoma. *Neurosurg Focus* 2014; **37**: 1–4.
8. Izumoto S, Tsuboi A, Oka Y, Suzuki T, Hashiba T, Kagawa N, et al. Phase II clinical trial of Wilms tumor 1 peptide vaccination for patients with recurrent glioblastoma multiforme. *J Neurosurg* 2008;

108: 963–971.

9. Manocha a, Jain S. WT1 in astrocytomas: comprehensive evaluation of immunohistochemical expression and its potential utility in different histological grades. *Indian J Cancer* 2019; **56**: 197–201.
10. Rauscher J, Beschoner R, Gierke M, Sotirios B, et al. WT1 expression increases with malignancy and indicates unfavourable outcome in astrocytoma. *J Clin Pathol.* 2014; **67**: 556–561.

Tables

Table 1. *WT1* gene expression as measured using IHC and RT-qPCR.

	WT1 Expression (IHC)	WT1 Expression (qPCR)	WT1 C _T mean	GDPH C _T mean	FCCT WT1	Rq
1	Overexpressed	Upregulated	39.47	32.14	3.42	3.42
2	Overexpressed	Upregulated	36	29.19	4.9	4.9
3	Partially expressed	Upregulated	35.01	31.7	55.62	55.62
4	Not expressed	Upregulated	35.64	28.89	5.42	5.42
5	Overexpressed	Upregulated	36.53	27.65	1.17	1.17
6	Overexpressed	Upregulated	34.98	31.21	40.55	40.55
7	Overexpressed	Down regulated	37.5	19.9	-361	0.0028
8	Not expressed	Down regulated	36.42	22.97	-20.25	0.05
9	Overexpressed	Down regulated	37.5	23.8	-24.49	0.04
10	Overexpressed	Down regulated	36.7	19.5	-280	2.76
11	Not expressed	Down regulated	37.47	28.08	-1.22	0.82
12	Overexpressed	Down regulated	38.95	21.3	-374	0.0027
13	Overexpressed	Down regulated	39.98	23.25	-197	0.0051

CT: threshold cycle (C_T), GAPDH: glyceraldehyde-3-phosphate dehydrogenase; Rq: Relative quantification.

Table 2. Patients data.

Overall (n=44)	
Age	
Mean (SD)	54.8 (15.3)
Range	11.0–82.0
Gender	
Female	18 (40.9%)
Male	26 (59.1%)
Tumour Location	
Frontal	16 (36.4%)
Occipital	3 (6.8%)
Parietal	17 (38.6%)
Temporal	8 (18.2%)
IDH1 Status	
IDH-mutant	18 (40.9%)
IDH-wildtype	26 (59.1%)
WT1 Expression (IHC)	
Not expressed	3 (6.8%)
Overexpressed	32 (72.7%)
Partially expressed	9 (20.5%)
Adjuvant Therapy	
Chemoradiotherapy	41 (93.2%)
None	3 (6.8%)
Chemotherapy Type	
Temozolomide	23 (52.3%)
Temozolomide Other	18 (41%)
Recurrence Interval	
Mean (SD)	579.3 (348.8)
Range	61.0 - 1344.0
Recurrence Time	

	Overall (n=44)
< one Year	28 (63.6%)
> one Year	16 (36.4%)

Table 3. McNemar test was used to detect matching compatibility between expression results obtained using IHC and qPCR.

WT1 expression measured by qPCR				
WT1 Expression (IHC)	Down regulated	Upregulated	Total	<i>P</i> -value
Down regulated	2	1	3	0.102 ^{x2}
Upregulated	5	5	10	0.221 ^{x2 c}
Total	7	6	13	

Sensitivity	83.3 %
Specificity	28.6 %
Accuracy	53.8 %

Figures

Figure 1

Schematic of the approach used in this study. The samples have been categorized based on *IDH1* mutation and their *WT1* expression. Recurrence interval used as determinant factor for patient's outcome.

Figure 2

IDH1 mutation status in glioblastoma using immunohistochemistry (IHC). IDH1 mutation showed positive expression while IDH1-wildtype showed negative expression). Scale bar, 100 μ m.

Figure 3

WT1 Expression using the IHC technique. Scale bar, 100 μ m. WT1 protein expression variation (Overexpression, partial expression, and no expression).

Figure 4

Recurrence interval among patients with glioblastoma and different IDH1 status and WT1 expression. The recurrence interval among patients with WT1 overexpression significantly differs between wildtype and mutant IDH1 (P-value < 0.048). This significance was not observed among cases with mutant IDH1 and partially expressed or overexpressed WT1 (P-value = 0.56) as well as among cases with wildtype IDH1 and WT1 partial or overexpression (P-value = 0.83).