

Sensitivity Assessment of Wilms Tumor Gene (*WT1*) Expression in Glioblastoma using qPCR and Immunohistochemistry and its Association with *IDH1* Mutation and Recurrence Interval

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Purpose: Wilms tumor 1 (*WT1*) gene has recently shown 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 the relationship between *WT1* expression, *IDH1* mutation and recurrence interval.

Patients and Methods: Clinical data were collected from 44 patients with glioblastomas, treated with adjuvant therapies. *WT1* expression was assessed in all cases using immunohistochemistry (IHC), while its gene expression was assessed in 13 clustered samples using polymerase chain reaction (qPCR). *IDH1* mutation was assessed using IHC. The sensitivity between IHC and RT-qPCR was examined. Kaplan–Meier curves were used to compare the recurrence-free interval (RFI) between *IDH1* and *WT1* expression groups.

Results: *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 3 cases. For the 13 cases tested by qPCR, 6 cases showed *WT1* upregulation and 7 cases showed *WT1* downregulation. 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 was significant. *IDH1*^{mutant} cases with *WT1* overexpression showed significant difference in RFI (p-value =0.048).

Conclusion: Parallel testing for *WT1* expression using IHC and qPCR is not reliable. However, IHC provides more accurate results. Moreover, *IDH1*^{mutant} glioblastomas with *WT1* overexpression are associated with late RFI particularly if temozolomide with additional chemotherapies are used.

Keywords: glioblastoma, *IDH1* mutation, *WT1* expression, chemotherapies, PCR sensitivity

Introduction

Wilms tumor 1 (*WT1*) gene encodes a zinc finger transcriptional factor that plays an important role in cell growth and differentiation.¹ *WT1* has been implicated in various malignancies. It was first identified as a tumor suppressor gene because of frequent chromosome 11p13 region deletions observed in childhood renal neoplasm and Wilms tumor and was then found to be overexpressed in leukemias and various solid tumors including breast and ovarian cancers^{2–4} Few studies have shown that *WT1* has a role in gliomagenesis.⁵ Consistently, *WT1* overexpression has been found in high-grade

gliomas.^{4,6,7} Recent clinical trials of cancer immunotherapy targeting *WT1* protein have shown promising results in glioblastomas, particularly in resistant cases. These results suggested that *WT1* is a possible target for immunotherapy in high-grade gliomas, which can increase the sensitivity of glioblastoma to chemoradiotherapy.⁸

The immunohistochemical approach is considered as the standard method to detect *WT1* protein expression in tumour cells. However, some studies have shown that *WT1* mRNA levels present results similar to that of the immunohistochemical score.⁴ Therefore, the most accurate method for testing *WT1* expression in glioblastoma is not obviously clear. Our study was designed to investigate whether IHC or qPCR is more sensitive for detecting *WT1* gene expression in glioblastoma cases.

Rauscher et al found that some high-grade gliomas lacked *WT1* expression, whereas Manocha et al identified an inverse relationship between *WT1* scores and *IDH1* mutation.^{8,9} They ascribed this negative expression in high-grade tumors to the younger age of patients and tumors possessing *IDH1* mutations.¹⁰ Our study was also designed to explore the relationship between *WT1* expression and *IDH1* mutation and how this influences tumour recurrence.

Patients and Methods

Sample Stratification

We included 44 patients with totally resected glioblastomas, who received adjuvant therapies, in the period 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). All patients involved in this study have provided informed consent. All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Histological diagnoses were made according to the World Health Organization (WHO) classification. Clinical data including age at diagnosis, gender, postoperative 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) was administered to all patients at the time of management. Some patients received additional chemotherapies including etoposide, bevacizumab, irinotecan, and lomustine.

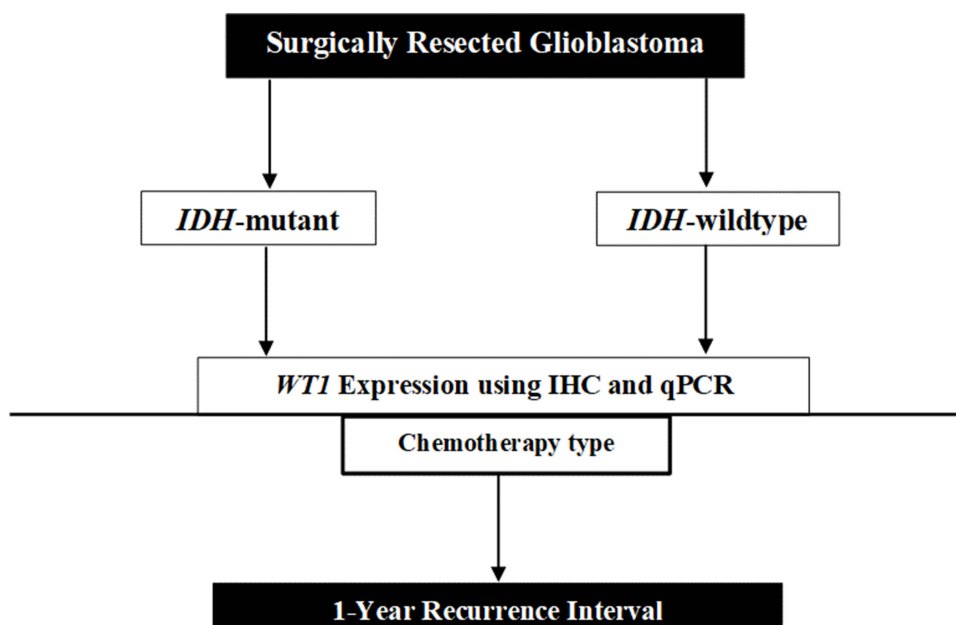


Figure 1 Schematic of the approach used in this study. The samples have been categorized based on *IDH1* mutation and their *WT1* expression. Recurrence interval used as determinant factor for patient's outcome.

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 (Dianova, Clone H09) mouse monoclonal antibody was performed with an OptiView detection kit on a BenchMark XT (Ventana). The assay procedure consisted of deparaffinization with EZ Prep at 75°C, heat pretreatment 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).

Assessment of WT1 Expression Through IHC and RT-qPCR

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* (Clone 6F-H2, Ventana) mouse monoclonal antibody was performed using the DAB detection kit on a BenchMark XT (Ventana). The assay procedure consisted of deparaffinization with EZ Prep at 75°C, pretreatment 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” by a certified neuropathologist.

Quantitative Analysis of WT1 Histochemical Expression on Glioblastomas

After immunostaining, a focal area of positive expression was evaluated under light microscopy using high-power (40×) magnification. Tumor cells were counted manually by a certified neuropathologist (MK). The labelling index was quantitatively assessed using the following equation: $\text{Labelling Index} = \frac{(\text{Staining-positive cytoplasm})}{(\text{Staining-positive cytoplasm} + \text{Staining-negative cytoplasm})}$ (Table 1). The staining pattern was categorized as 1) over-expressed, 2) focal expressed, 3) partially expressed and 4) none-expressed (Figure 3).

Assessment of WT1 Gene Expression Using Reverse Transcriptase-qPCR

H&E-stained sections from FFPE tissue blocks were examined by a neuropathologist (MK) to select regions from which RNA could be extracted. RNA was isolated by standard procedures from selected tissue fragments containing a high percentage of tumor cells. RNA extraction was performed using FFPE RNeasy Kit (no. 73504) according to the manufacturer’s instructions. CDNA was synthesized by ImProm-II™ Reverse Transcription System (CAT no. A3800). Quantitative-PCR was performed by QuantiFast SYBR® Green RT-PCR Kit (no. 204154) with two primer

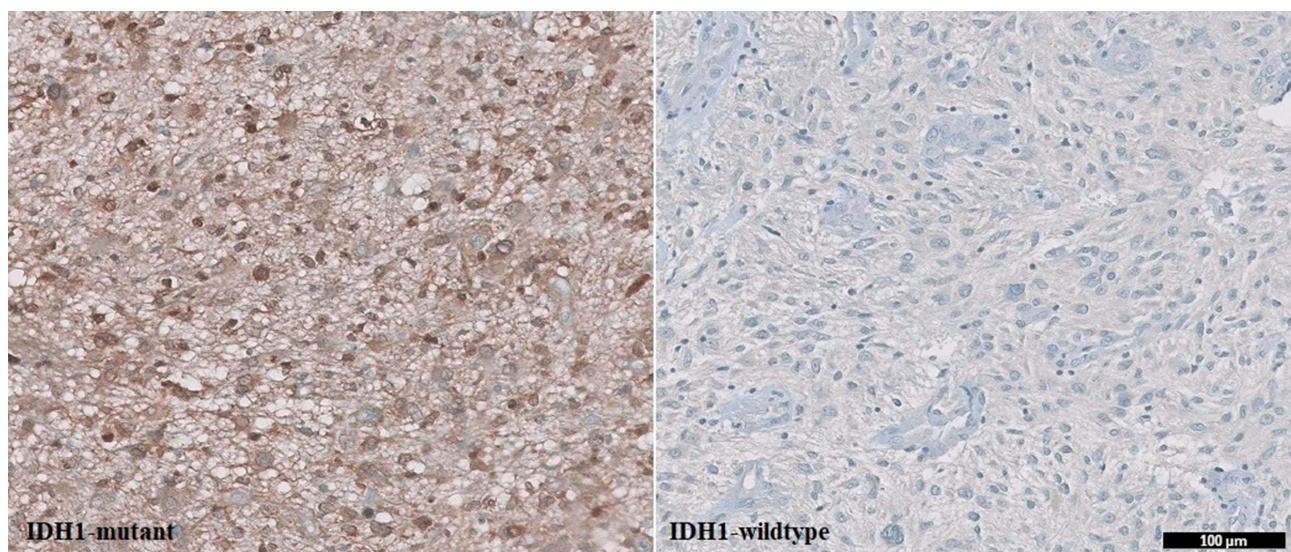


Figure 2 IDH1 mutation status in glioblastoma using immunohistochemistry (IHC). IDH1 mutation showed positive expression while IDH1-wildtype showed negative expression). Scale bar, 100 µm.

Table 1 The Labelling Index (%) Was Assessed Through the Following Scoring System

Expression	Labelling Index (%)
No expression	0
Focal expression	>0–20
Partial expression	>20–50
Diffuse expression	>50

Note: For statistical analysis, the scores were divided by 100.

pairs, the targeted gene: *WT1* and the reference gene: *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) (Table 2). The Annealing Temperature was 58°C. Of the 44 enrolled glioblastoma cases, only 13 samples had adequate RNA quality for RT-PCR. This limitation should be taken into consideration during assessment of *WT1* expression in pathological practice. The samples were divided into case (n = 13) and control (n = 2) groups. The control cases were low-grade glioma and non-glioma. IHC results showed no *WT1* expression in control cases. 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 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) $\Delta\Delta C_T = \Delta C_T$ test sample – ΔC_T control sample, (3) Relative quantification (Rq) = $2^{-\Delta\Delta C_T} = \text{value}^*$. The fold change (differential expression) was also calculated. ΔC_T values for each sample were determined using the following formula: $\Delta C_T = [\text{mean } C_T \text{ reference gene} - \text{mean } C_T \text{ target gene}]$; however, FC-WT1 >0 represents upregulation, whereas FC-WT1 <0 represents downregulation of gene (Table 3).

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 completely 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 locations and tumors in these locations were observed in 33 cases (75%). IDH1^{wildtype} was found in 26 cases (59.1%) and the remaining 18 cases (40.9%) were IDH1^{mutant}. IHC revealed *WT1* overexpression in 32 cases (72.7%), partial expression in 9 cases (20.5%), and no expression in 3 cases (6.8%). For the 13 cases in which *WT1* expression was tested by qPCR, 6 and 7 cases had up- and downregulated *WT1* expression, respectively (Tables 3 and 4). For post-surgical treatment, 41 patients (93.2%) received chemoradiotherapy and 3 patients did not receive any adjuvant therapies. 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 interval was 579 days after the total surgical resection of the tumor. Approximately, 36% (n = 16) of the patients had tumor recurrence after 1-year of resection while 63.6% (n = 28) showed recurrence before 1-year of resection. Table 4 summarizes the descriptive distribution of the data.

IHC and RT-qPCR Detection of *WT1* Expression in Patients with Glioblastoma

There was no clear evidence in the literature whether IHC or qPCR showed better results when measuring *WT1* expression. RT-qPCR can be processed using fragmented tissue, but the RNA content is often not enough to provide beneficial results. Therefore, we investigated the accuracy of both methods assuming that IHC, based on the previous published data, is more accurate.

Tumour samples (n=31) with low RNA concentration <20 nM have been excluded from qPCR test. IHC and qPCR were used in the 13 clustered samples that had RNA concentrations >20 nM. Two cases showed *WT1* downregulation (no expression) in both IHC and qPCR. A single case, with low RNA concentration (22.7 gM) showed no *WT1* expression by IHC but *WT1* upregulation by qPCR. The five cases, that showed *WT1* overexpression on IHC, showed downregulation by qPCR. The

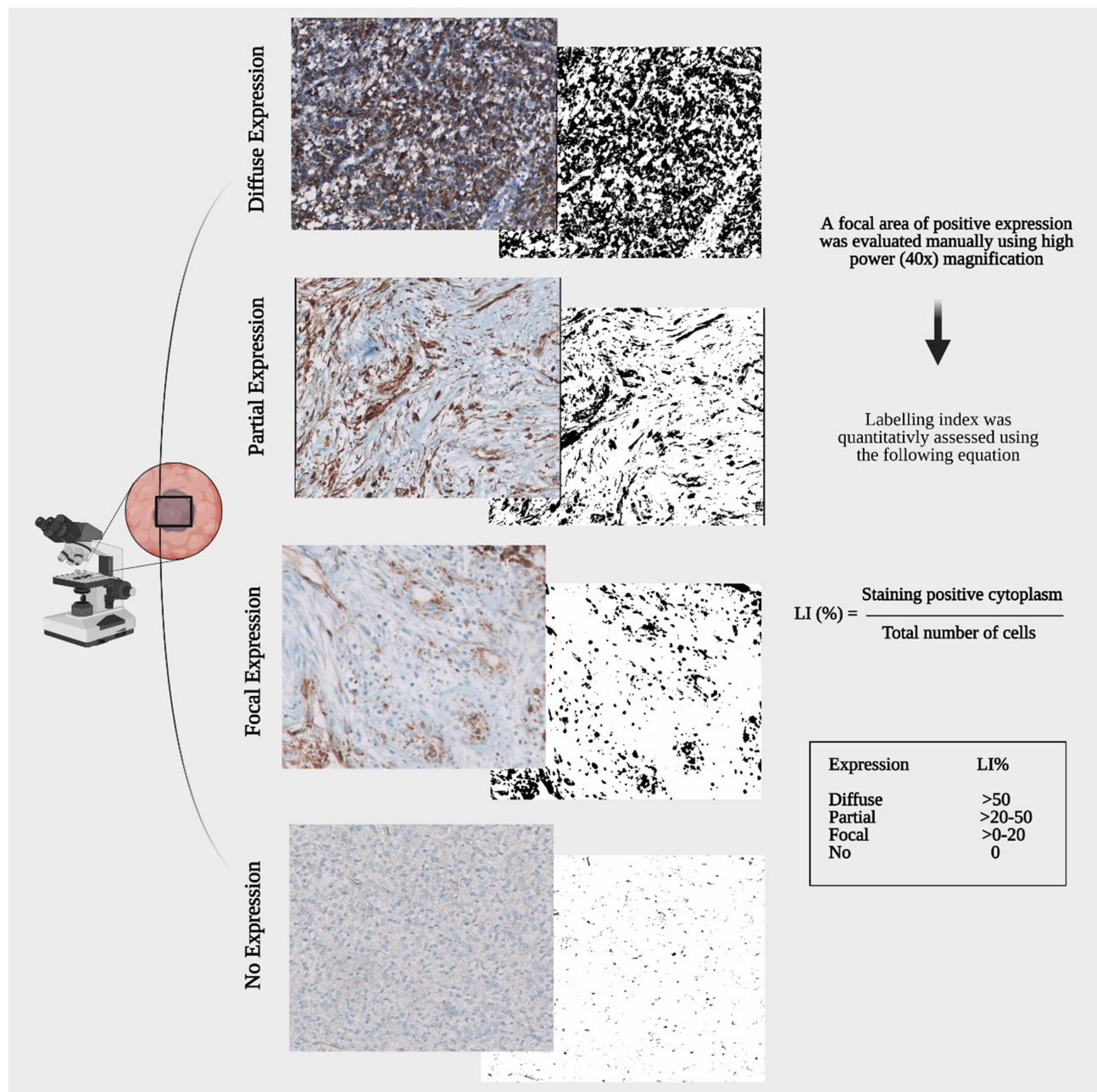


Figure 3 An algorithm of analysis workflow describing the quantitative assessment of WT1 expression through IHC. The expression categories are based on labelling indices (%).

remaining five cases had parallel results. There was no statistically significant difference in *WT1* expression 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 5). The lack of significant difference in *WT1* expression between IHC and qPCR indicates that both methods are not reliable to be used in parallel.

Relationship Between IDH1 Status, WT1 Expression, and Recurrence Interval

The recurrence interval among patients with *WT1* overexpression significantly differs between cases with wild-type and mutant IDH1 (P -value=0.048). IDH1^{mutant} cases showed late recurrence, after 1-year (Figure 4A). This significance was not observed among IDH1^{mutant} cases with partially expressed or overexpressed *WT1* (P -value = 0.56) (Figure 4B). These results indicate that in cases with mutant

Table 2 Primers for *WT1* Gene Expression Analysis

Primer	Sequence
WT1-Forward	CACACGCACGGTGTCTTC
WT1-Reverse	AGATGCCGACCGTACAAG
GAPDH-Forward	CCCCCAATGTATCCGTTGTG
GAPDH-Reverse	TAGCCCAGGATGCCCTTAGT

Abbreviation: GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

IDH1, *WT1* upregulation lowers the chance of tumor recurrence. No statistically significant difference in recurrence interval was observed among IDH1^{wildtype} cases with *WT1* partial or overexpression (P -value = 0.83) (Figure 4C).

Moreover, IDH1^{mutant} glioblastomas with *WT1* overexpression, who received TMZ with additional chemotherapies, showed late recurrence intervals than those who received TMZ alone (P -value = 0.049). On the other hand, IDH1^{wildtype} glioblastomas with *WT1* overexpression who received TMZ or TMZ with additional chemotherapies showed no significant difference in RFI (P -value = 0.19) (Figure 5A and B)

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, the current standard treatment for patients with glioblastoma is 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.

Table 4 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%)
Other	18 (41%)
Recurrence Interval	
Mean (SD)	579.3 (348.8)
Range	61.0–1344.0
Recurrence Time	
<1 Year	28 (63.6%)
>1 Year	16 (36.4%)

Table 3 *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	Downregulated	37.5	19.9	−361	0.0028
8	Not expressed	Downregulated	36.42	22.97	−20.25	0.05
9	Overexpressed	Downregulated	37.5	23.8	−24.49	0.04
10	Overexpressed	Downregulated	36.7	19.5	−280	2.76
11	Not expressed	Downregulated	37.47	28.08	−1.22	0.82
12	Overexpressed	Downregulated	38.95	21.3	−374	0.0027
13	Overexpressed	Downregulated	39.98	23.25	−197	0.0051

Abbreviations: CT, threshold cycle (C_T); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FCCT, fold change cycle threshold; Rq, relative quantification.

Table 5 McNemar Test Was Used to Detect Matching Compatibility Between Expression Results Obtained Using IHC and qPCR

WT1 Expression Measured by qPCR				
WT1 Expression (IHC)	Downregulated		Upregulated	Total P-value
	Downregulated	2	1	
Upregulated	5	5	10	0.221 ^{x2 c}
Total	7	6	13	
Sensitivity	83.3%			
Specificity	28.6%			
Accuracy	53.8%			

WT1 encodes a zinc finger transcriptional factor, which has been implicated in various malignancies such as childhood renal neoplasm (WT), leukemias, breast and ovarian

cancers.¹⁻⁴ Recent studies have shown that WT1 plays a role in gliomagenesis.⁵ Its 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 treatment planning 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, which increases the sensitivity of glioblastoma to chemotherapy.⁸

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 was no significant difference in WT1 expression by using different tests (IHC or qPCR) with different RNA concentrations.

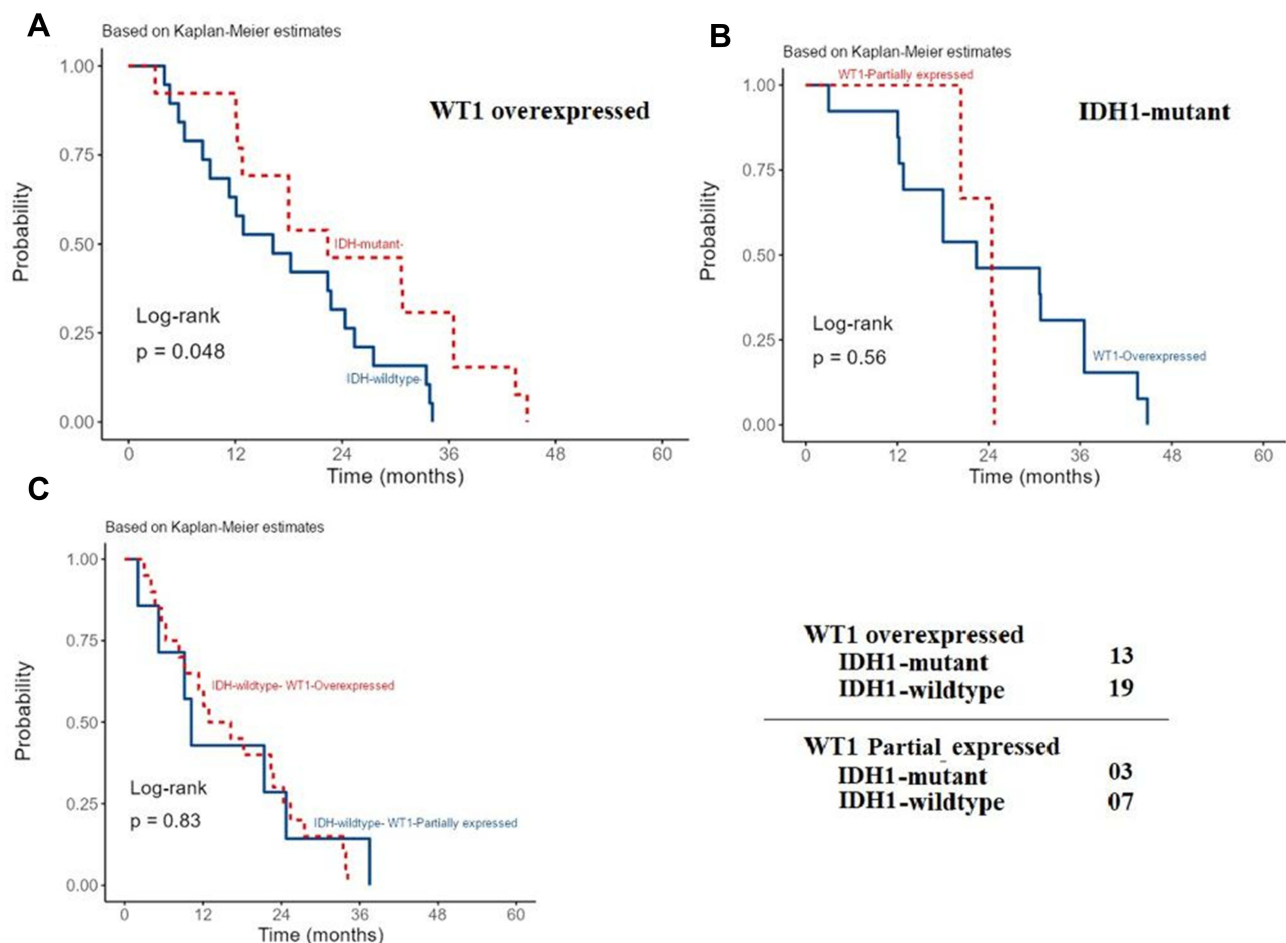


Figure 4 Recurrence interval among patients with glioblastoma and different IDH1 status and WT1 expression. The recurrence interval among patients with WT1 overexpression significantly differs between wild-type and mutant IDH1 (P-value < 0.048) (A). This significance was not observed among cases with mutant IDH1 and partially expressed or overexpressed WT1 (P-value = 0.56) (B) as well as among cases with wild-type IDH1 and WT1 partial or overexpression (P-value = 0.83) (C).

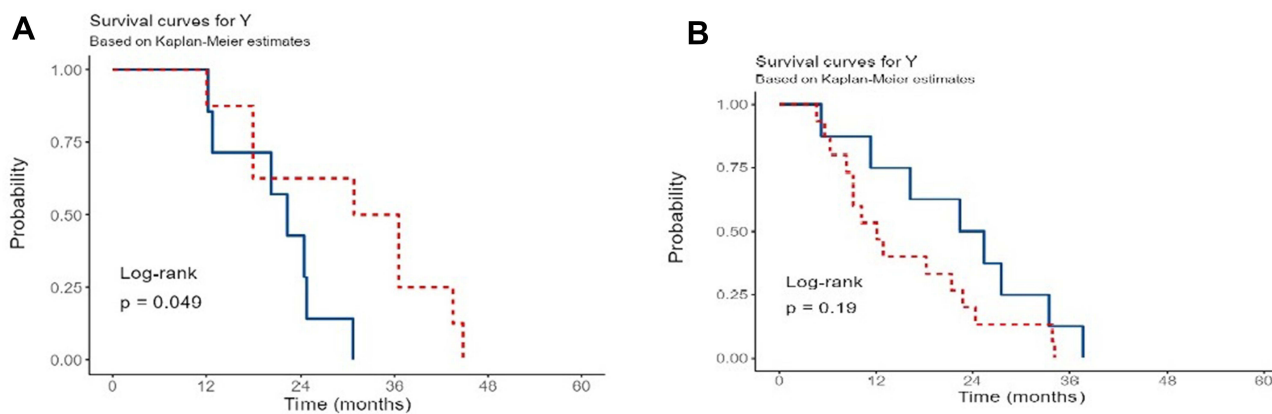


Figure 5 The recurrence interval among glioblastoma patients, with *WT1* overexpression and different *IDH1* status, who received different treatment modalities. **Notes:** (A) The association between chemotherapies and *IDH1* mutation (blue curve: *IDH1* mutant with TMZ; red curve: *IDH1* mutant with only TMZ and other chemotherapies). (B) The association between chemotherapies and wildtype *IDH1* (blue curve: *IDH1* wildtype with TMZ and other chemotherapies; red curve: *IDH1* wildtype with only TMZ).

However, low RNA volume and concentration may give false qPCR results. This does not occur when *WT1* expression being tested by IHC. The proteins detected by IHC are more stable and well preserved after tissue processing. Indeed, it gives more accurate and sensitive results. On the other hand, the RNAs detected by Rt-PCR are not stable chemically and mostly degraded after tissue processing, especially FFPE tissue. 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 showed that parallel testing of *WT1* expression using IHC and qPCR was not reliable; thus, IHC provides more accurate results than qPCR.

The association between *IDH1* mutation and *WT1* expression has also not been thoroughly investigated. Manocha et al identified an inverse relationship between *WT1* scores and *IDH1* mutation.⁹ Rauscher et al found that some anaplastic astrocytomas and glioblastomas 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 TCGA dataset, 12 glioblastoma cases have been investigated. All the cases were *IDH1*^{wildtype} while six cases had *WT1* upregulated and six downregulated. The effect of *WT1* expression on tumor recurrence interval was insignificant (p -value = 0.149). In our analysis, we found that *IDH1*^{mutant} glioblastomas with *WT1* overexpression are associated with late tumor recurrence interval compared with cases with *IDH1*^{wildtype}. 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, to increase the sensitivity to TMZ, and to decrease tumor recurrence.

Conclusion

Parallel testing for *WT1* expression using IHC and qPCR is not reliable. However, IHC provides more accurate results. Moreover, *IDH1*-mutant glioblastomas with *WT1* overexpression are associated with late RFI compared to *IDH1* wild-type cases, particularly if temozolomide with additional chemotherapies are used.

Code Availability

N/A.

Data Sharing Statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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). All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Consent to Participate

All contributors have given consent to participate in the study.

Author Contributions

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 SH, data interpretation All authors contributed to data analysis, drafting or revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure

All authors reported no conflicts of interest for this work.

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