Research Article
Longevity-Related Gene Transcriptomic Signature in Glioblastoma Multiforme

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Glioblastoma multiforme (GBM) (grade IV astrocytoma) has been assumed to be the most fatal type of glioma with low survival and high recurrence rates, even after prompt surgical removal and aggressive courses of treatment. Transcriptional reprogramming to stem cell-like state could explain some of the deregulated molecular signatures in GBM disease. The present study aimed to quantify the expression profiling of longevity-related transcriptional factors SOX2, OCT3/4, and NANOG to evaluate their diagnostic and performance values in high-grade gliomas. Forty-four specimens were obtained from glioblastoma patients (10 females and 34 males). Quantitative real-time polymerase chain reaction was applied for relative gene expression quantification. In silico network analysis was executed. NANOG and OCT3/4 mRNA expression levels were significantly downregulated while that of SOX2 was upregulated in cancer compared to noncancer tissues. Receiver operating characteristic curve analysis showed high diagnostic performance of NANOG and OCT3/4 than SOX2. However, the aberrant expressions of the genes studied were not associated with the prognostic variables in the current population. In conclusion, the current study highlighted the aberrant expression of certain longevity-associated transcription factors in glioblastoma multiforme which may direct the attention towards new strategies in the treatment of such lethal disease.

1. Introduction

Tumors of the brain were considered one of the ten most common causes of cancer-related mortality [1]. According to the World Health Organization (WHO) classification, the primary brain tumors are categorized into glial tumors (e.g., glioblastoma, astrocytomas, oligodendrogial tumors, and ependymal tumors), embryonic tumors (e.g., medulloblastomas), tumors of the meninges, tumors of the hematopoietic system, and tumors of the sellar region [2]. The most fatal type of glioma has been reported to be the glioblastoma multiforme (GBM) [3] which represents up to 50% of almost all primary brain gliomas [4] with poor prognosis [5] and median survival rate of nearly 25 months after treatment [6]. The recurrence of the tumor after prompt surgical removal despite the aggressive courses of radio- and chemotherapy denotes the limited understanding of the disease biology [7]. Dell’Albani has stated that “new insights into the causes and the potential treatment of CNS tumors have come from disclosing relations with genes that regulate cell
growth, proliferation, differentiation, and death during nor-
mal development” [7]. These genes may represent a new tar-
get for GBM treatment by ameliorating the survival rate and
preventing or minimizing disease recurrence.

Several emerging evidences support the reactivation of
pluripotent transcription factors in many types of cancer
[8–12]. As a normal biological phenomenon, these factors
are expressed in embryonic stem cells (ESCs) and somatic
cells where they imply the self-renewal [13] and the pluripo-
tency characteristics [14]. As cancer development is a multi-
step process in which differentiated cells transform into
immature ones, these factors could participate in cancer
biogenesis and/or progress.

Among these pluripotent transcription factors overex-
pressed in high-grade gliomas are “sex-determining region
Y-Box (SOX2), octamer-binding transcription factor 4
(OCT 4), and Nanog homeobox (NANOG)” [13, 15].

SOX2 gene encodes a transcriptional factor (TF) of 317
amino acids which contains a high-mobility group DNA-
binding domain (Figure 1(a)) [16]. It implicated in embry-
onic development regulation, cell fate determination, and
embryonic stem cell pluripotency. More specifically, it was
reported to control the neural stem cell proliferation and
differentiation into neurons, astrocytes, or oligodendrocytes
[17]. SOX2 is expressed in stem cells of endoderm-derived
organs such as the liver, pancreas, and stomach [18], and
its aberrant expression has been found to support self-
renewal and inhibit neuronal differentiation [19]. Addition-
ally, SOX2 knockout in glioblastoma stem cells isolated from
human glioma tumor inhibits cell proliferation and tumori-
genicity in immunodeficient mice [20].

OCT3/4 is a member of a transcription POU family (Figure 1(b)) which has to react with other TFs in order
to stimulate or inhibit gene expression [21] in ESCs
through heterodimerization with SOX2. It was implicated in embryonic development regulation, cell fate determina-
tion, and embryonic stem cell pluripotency [22]. Finally,
NANOG (Figure 1(c)) is involved in gene regulation with
the aforementioned two transcription factors through their
binding to the promoters of several genes which mediates
the pluripotency, inhibits embryonic stem cell differenta-
tion, and autorepresses its own expression in differentiating
cells [22]. It has been found to be localized mainly in the
corei of high-grade glioma cells than lower grades [15].
Despite the fact that OCT3/4 and NANOG have shown a
direct relationship with the tumor grade, their oncogenic
nature in brain tumorigenesis has not been established
yet [23].

Up to our knowledge, there were no previous studies
that relate the expression of the aforementioned longevity-
related transcription factors in GBM patients among the
Arab population. Hence, the present study for the first time
aimed to quantify the expression levels of these markers in
GBM sample of Egyptian patients and to correlate their
expressions with the available clinicopathological features.
A thorough understanding of the relevance of each bio-
marker in GBM will be in need not only for reliable diagno-
sis of the disease but also to participate in future drug design
for this fetal tumor.

2. Materials and Methods

2.1. Study Participants and Tissue Samples. The current study
included 44 glioblastoma patients (10 females and 34 males,
aged 38 to 62 years) assessed retrospectively from archived
formalin-fixed paraffin-embedded section (FFPE) specimens
of the Pathology Department, Mansoura University Hospi-
tals, Egypt, from 2010 to 2013. They had glioblastoma
multiforme grade 4, subjected to surgical removal and post-
operative irradiation, and followed up for more than 36
months. Specimens were collected before receiving chemo-
therapy or radiotherapy prior to surgery. They were com-
pared to 10 FFPE noncancerous brain specimens obtained
from patients undergoing brain tissue resection for other
reasons collected from the same hospital. Guidelines in the
Declaration of Helsinki were followed, and an approval by
the Medical Research Ethics Committee of Faculty of Medi-
cine, Suez Canal University, was obtained before taking part.
Written informed consent was obtained from all participants
before providing the archived tissue samples as part of their
routine register in our University Teaching Hospitals.

2.2. RNA Extraction. Extraction of total RNA from FFPE
specimens was done using RNeasy FFPE Kit (Qiagen,
52304) according to the protocol of the manufacturer.
RNA concentration and purity were assessed with Nano-
Drop ND-1000 spectrophotometer (NanoDrop Tech. Inc.,
Wilmington, DE, USA), followed by agarose gel electropho-
resis (1%) check for RNA integrity.

2.3. Reverse Transcription (RT). Complementary DNA
(cDNA) was obtained by total RNA conversion using the
High-Capacity cDNA Reverse Transcription Kit (Applied
Biosystems, P/N 4368814) with RT random primers on
T-Professional Basic, Biometra PCR System (Biometra,
Goettingen, Germany), as previously described [12]. Approp-
riate negative and positive controls were included in
each experiment.

2.4. Gene Expression Profiling. The Minimum Information
for Publication of Quantitative Real-Time PCR Experi-
ments (MIQE) guidelines were followed for the real-time
PCR reactions. Pluripotent gene relative expressions were
assessed using “Universal PCR master mix II, No UNG
(2x)” (TaqMan®, Applied Biosystems, P/N 4440043), Taq-
Man assay (Applied Biosystems, assay ID Hs00427620_m1
for OCT3/4) and compared to the endogenous control
TATA box binding protein (TBP) (Hs00427620_m1) which
has been proved in our previous work [24] to be uniformly
and stably expressed with no significant difference between
GBM and noncancer tissues for gene expression normaliza-
tion. PCRs were done in 20 μl total volume using “StepOne™
Real-Time PCR System (Applied Biosystems)” as previously
described in details [25].

2.5. Statistical Analysis. Data analysis was done using PC-
ORD ver. 5 software package and Statistical Package for the
Social Sciences (SPSS) for windows software (version 22.0).
Two-tailed statistical tests were used for continuous and
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(a)

SOX2

High mobility group box domain

Transcription factor SOX

(b)

OCT3/4

POU-specific domain

DNA-binding domain

Figure 1: Continued.
categorical variables. Correlation analysis between the variables was performed via Pearson’s correlation coefficient. A p value < 0.05 was considered significant. The fold change of mRNA expression in each patient’s cancer tissue relative to the mean of controls was calculated using Livak method that depends on the quantitation cycle (Cq) value with the following equation:

\[ \text{relative quantity} = 2^{-\Delta\Delta C_q} \] [26].

The diagnostic performance of pluripotent genes was evaluated by receiver operating characteristic (ROC) analysis. Kaplan–Meier estimator was generated for survival analysis, and log-rank test was applied for different Kaplan–Meier curve (stratified by clinicopathological features) comparisons. Linear regression analysis using ENTER method was performed to evaluate potential factors affecting the overall survival of patients. Two-way Hierarchical cluster analysis was run for exploratory multivariate analysis. Ward’s method and Euclidean (Pythagorean) were adjusted for linkage method and distance measure, respectively, with a beta value of −0.75 to reach the minimum % of chaining.

3. Results

3.1. Expression Profile of Pluripotent Genes. Baseline clinical features of the study participants are illustrated in Table 1. Relative expression analyses of pluripotent genes in brain cancer specimens were compared to TBP. Our results revealed that the expression levels of NANOG and OCT3/4 were significantly downregulated (p < 0.001 and = 0.001, resp.) while that of SOX2 was significantly upregulated (p = 0.0027) in tumor specimens compared to noncancer tissues (Figures 2(a) and 2(b)).
and OCT3/4 mRNAs showed high diagnostic values as biomarkers for GBM (AUC = 0.886 ± 0.054 and 0.736 ± 0.078, resp.) (Figure 3).

3.2. Association with Clinicopathological Characteristics and Survival Analysis. Higher OCT3/4 gene expression was noted in elder GBM patients (p = 0.036). No statistically significant association was found with any other parameters (Figure 4). Correlation analysis revealed moderate correlation between NANOG and SOX2 gene expression profile (r = 0.484, p = 0.023). In addition, elder age of patients was associated with poor overall survival (OS) (r = −0.479, p = 0.024) and disease-free survival (DFS) (r = −0.481, p = 0.023) (Figure 5).

Linear regression analysis was performed to evaluate potential factors affecting overall survival of patients. None of the genes or clinicopathological variables was determined as a good prognostic marker for patients’ survival in the study population (Table 2). However, survival analysis in GBM by log-rank and Tarone-Ware tests showed poor OS among elder patients (Figure 6 and Table S1).

3.3. Multivariate Analysis. Exploratory multivariate analysis by principle component and hierarchical cluster analyses classified patients into 3 groups based on the relative expression of the combined genes (Figure 7). However, there was no clear demarcation found between patients according to age, gender, tumor site, and recurrence (Figure S1).

4. Discussion

The presence of a significant heterogeneity in certain types of solid tumors including GBM is becoming obvious. Hence, it will be rational to search for and evaluate specific molecular markers that could assist in diagnosis and/or prognosis of these tumors and could act as targeted molecular markers for personalized therapy [7]. Here, we attempted to investigate the presence of a molecular signature of longevity-related genes (SOX2, NANOG, and OCT3/4) by examining their mRNA expression in GBM tissues relative to noncancer tissues. Our analyses revealed that the expression level of SOX2 was significantly upregulated. This finding was consistent with several independent cohorts [28–30] and in part with Guo et al., [13] who detected an overexpression of

| Variables                  | Number (%) or mean ± SE |
|----------------------------|-------------------------|
| Age                        | 51.4 ± 0.97             |
| Age categories             |                         |
| 35–50 y                    | 18 (40.9)               |
| >50 y                      | 26 (59.1)               |
| Gender                     |                         |
| Female                     | 10 (22.7)               |
| Male                       | 34 (77.3)               |
| Tumor site                 |                         |
| Frontal                    | 22 (50)                 |
| Frontotemporal             | 4 (9.1)                 |
| Temporoparietal            | 18 (40.9)               |
| Recurrence                 |                         |
| Nonrecurrent               | 36 (81.8)               |
| Recurrent                  | 8 (18.2)                |
| Disease-free survival (months) |                     |
| Mean ± SE                  | 15.1 ± 0.85             |
| Range                      | 6–27                    |
| Prolonged DFS (>1 y)       | 28 (63.6)               |
| Short DFS (≤1 y)           | 16 (36.4)               |
| Overall survival (months)  |                         |
| Mean ± SE                  | 15.6 ± 0.86             |
| Range                      | 8–27                    |
| High survival (>1 y)       | 30 (68.2)               |
| Low survival (≤1 y)        | 14 (31.8)               |

Figure 2: Expression profile of pluripotent genes in GBM patients compared to controls. (a) Values are presented as medians and quartiles of fold change relative to controls. The box defines upper and lower quartiles (25% and 75%, resp.) and the Whisker bars indicate upper and lower adjacent limits. TBP was used as an internal control. Noncancer tissues was set to have a relative expression value of 1.0. Mann–Whitney U test was used for comparison. p value < 0.05 was considered statistically significant. (b) Frequency of patients with up- and downregulated genes.
SOX2 mRNA in grade IV gliomas compared to grade II. Of the three longevity-related factors, SOX2 seems to be the playmaker in the development of brain tumors [18]. When overexpressed, it promotes cell cycle progression into S phase and proliferation [3, 20, 28, 31], which were attenuated by application of SOX2-RNAi (RNA interference) therapy [32]. At the cellular level, Garros-Regulez et al. [33] proposed SOX2 upregulation via activation of GBM-specific signaling pathways that maintain the overexpression of SOX2 via transforming growth factor-beta (TGF-β), Sonic Hedgehog (SHH), epidermal growth factor receptor (EGFR), and fibroblast growth factor receptor (FGFR)

Figure 3: Diagnostic performance of pluripotent genes to discriminate between GBM and noncancer samples. NANOG and OCT3/4 showed high diagnostic values as biomarkers for GBM.
Figure 4: Association between gene expression and the clinicopathological features. Values are presented as medians and quartiles of fold change relative to controls. The box defines upper and lower quartiles (25% and 75%, resp.) and the whisker bars indicate upper and lower adjacent limits. TBP was used as an internal control. Noncancer tissues were set to have a relative expression value of 1.0. Mann–Whitney U and Kruskal-Wallis tests were used for comparison. p value < 0.05 was considered statistically significant. F: frontal tumor site; FT: frontotemporal; TP: temporoparietal; R: recurrent; NR: nonrecurrent.

Despite that our in silico analysis revealed that the expression of the studied stem-related factors has similar colocalization and physical interactions with each other [12], they seem to be differentially expressed independently in the current samples. We found that NANOG and OCT3/4 were significantly downregulated in GBM tissues. Our finding might seem contradictory to the stemness role these pluripotent transcription factors play; however, it is worth to emphasize that the mechanistic functions of SOX2, OCT4, and NANOG in cancer cells are a little different in each stage of tumor progress. Kallas et al. reported high levels of SOX2, OCT4, and NANOG transcription factor expressions at the beginning of their tested human embryonic stem cell differentiation. However, on progress of the differentiation process, a decline in OCT4 and NANOG expression levels was observed, while expression of SOX2 was kept at a high level [35]. They suggested that the pluripotency is maintained by a transcriptional network that is harmonized by the aforementioned core transcription factors. During differentiation, the epigenetic modifications could play a role in level modulation of these factors.

The other possible reasons for inconsistency of gene expression for the three stem cell marker studies could be sampling bias and/or relatively low expression levels of these pathways. In addition, SOX2 gene amplification and DNA promoter hypomethylation have been reported in a group of GBM patients to expand the mechanism responsible for SOX2 upregulation [34].

Figure 5: Correlation matrix between transcriptomic signature and the clinicopathological features. Pearson’s correlation analysis was performed and represented as color gradient.

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factors within the individual GBM tissue examined in the current study [36]. This could be explained by the unique stem cell signature that has been implied by each tumor due to the inherent intratumor heterogeneity within GBM tissues [37–39]. Our multivariate analysis and the hierarchical cluster analysis confirmed the previous suggestions by revealing classification of the study population into 3 groups based on the combined gene expression that confirm a specific protumorigenic profile. Similar to other combinations of cancer stem cell markers in other types of cancer [40, 41], previous studies revealed that cancer stem cells which were isolated using different markers in the same

### Table 2: Linear regression analysis to determine predictors for survival.

|                     | Unstandardized coefficients | Standardized coefficients | 95% confidence interval for B |
|---------------------|-----------------------------|---------------------------|-------------------------------|
|                     | B   | Std. error | Beta | t    | Sig. | Lower bound | Upper bound |
| (Constant)          | 34.675 | 10.593 |       | 3.273 | 0.006 | 11.955 | 57.395 |
| Age                 | −0.318 | 0.220 | −0.363 | −1.448 | 0.170 | −0.790 | 0.153 |
| Gender              | −2.142 | 3.510 | −0.170 | −0.610 | 0.551 | −9.671 | 5.387 |
| Tumor site          | 0.079 | 1.498 | 0.014 | 0.053 | 0.959 | −3.134 | 3.292 |
| Recurrence          | 0.501 | 3.635 | 0.036 | 0.138 | 0.892 | −7.295 | 8.296 |
| NANO2               | 0.374 | 3.095 | 0.033 | 0.121 | 0.905 | −6.263 | 7.011 |
| OCT3/4              | −0.943 | 1.339 | −0.173 | −0.705 | 0.493 | −3.815 | 1.928 |
| SOX2                | −0.085 | 0.123 | −0.214 | −0.691 | 0.501 | −0.348 | 0.178 |

**Figure 6:** Kaplan–Meier survival curve in GBM patients. Log-rank (Mantel-Cox) test was used for comparison. Statistical significance at *p* < 0.05.
cancer phenotype had different expression profiles quantified by real-time PCR. Combined expression analysis might more accurately identify true cancer stem cells for each type of cancer [40], including GBM tumors.

Ji et al. reported that unlike normal stem cells, OCT4 could be dispensable for self-renewal, survival, and differentiation of transformed cells. They provided direct evidence for the functional divergence of OCT4 from the pluripotent state following the cancer tissue transformation [42]. This could support the downregulation of this stem-related marker noted in the current advanced stages of GBM cases. Additionally, Bradshaw et al. [36] reported low OCT4 relative expressions at the transcription and protein levels within their FFPE GBM samples. They speculated that the relatively OCT4-expressing cell low number could indicate the most primitive stem cell population within GBM which may possibly bring about the rest of downstream cells within the GBM tumor. Otherwise, the SOX2 ubiquitous redundancy is more likely to be expressed in the more differentiated cells reflecting its usefulness as a potential progenitor cell marker within the GBM tissues [36].

In contrast to the finding of Zbinden et al. [43] that NANOG was essential for GBM tumourigenicity in orthotopic xenografts, we found downregulation of this marker in the current GBM samples. We speculated that this difference could be due to either the low NANOG-expressing cell number within the study samples as mentioned above for the OCT4 marker or the type of NANOG transcript that has been quantified by the available quantitative PCR analysis at the time of the current work which preferentially recognized the varying levels of NANOG expression. As NANOG is coded by two genes (i.e., NANOG and NANOGP8) in

Figure 7: Multivariate analyses cluster GBM patients according to transcriptomic signature. PC-ORD v5.0 was used for exploratory multivariate analysis. Data set was profiled by the program. There was no need for transformation as beta diversity was zero and there was no outlier. (a) Ordination graph by PCO and (b) two-way hierarchical cluster analysis. The following parameters were adjusted: linkage method; Ward’s method; distance method; Euclidean method; relativizing matrix by column maximum; and matrix coding percentile by column. Percent chaining = 7.38. Clustering identified three patient groups according to their gene expression. The red clade for overexpression of the three pluripotent genes, the green clade discriminates patients with gene downregulation, and the blue clade has variable degrees of expression. Two samples (black clade) were out-group from the other clusters.
human, it has been found that NANOGP8 is the most abundantly expressed of the two NANOG-encoding genes in GBMs, accounting for more than ninety percent of all NANOG-encoding mRNAs in a number of previously tested cases [43]. However, future lineage analyses will be required for unravelling the high NANOG-expressing cell nature and NANOG expression stability as recommended by the latter researchers. Correlating the available clinicopathological features including the survival data of GBM cases with the gene expression results revealed that poor overall survival and disease-free survival were found significantly among patients as reported by previous studies [44, 45]. Despite that GBM can occur in individuals of any age according to the previous population-based studies, the median age is nearly above 60 years. Additionally, primary GBMs have been reported to develop commonly in older individuals (mean, 55 years), whereas secondary ones were found in middle-aged subjects (39 year olds) [4].

5. Conclusion

The current study findings highlighted the dysregulated longevity-related gene expression in GBM Egyptian cases that could have a potential role in carcinogenesis and procurement of stemness-like properties in this type of tumors. The current study could be limited by the relatively small sample size and the fact that all patients have grade IV gliomas, although this last issue increases the specificity of the study results that confined to one stage of GBM. Additional large-scale studies including different glioma grades are recommended to evaluate the relation of the studied longevity-related gene expression with different WHO grades as well as to confirm their putative role as diagnostic and/or prognostic biomarkers. These could be an interesting era for future individualized molecular-targeted therapy for GBM patients.

Abbreviations

Cq: Quantitation cycle
DFS: Disease-free survival
ESCs: Embryonic stem cells
GBM: Glioblastoma multiforme
MIQE: Minimum Information for Publication of Quantitative Real-Time PCR Experiments
NANOG: Nanog homeobox
OCT: Octamer-binding transcription factor
OS: Overall survival
ROC: Receiver-operating characteristic
RT: Reverse transcription
SOX2: Sex-determining region Y-Box.

Conflicts of Interest

The authors declare that they have no competing of interests.

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Supplementary Materials

Table S1: Kaplan–Meier curves comparing survival of patients with different clinical variables. Figure S1: multivariate analysis stratified by clinicopathological features. (Supplementary Materials)

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