Immunoexpression of p62/SQSTM1/Sequestosome-1 in human primary and recurrent IDH1/2 wild-type glioblastoma: A pilot study

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Received May 27, 2022; Accepted June 30, 2022

DOI: 10.3892/ol.2022.13456

Asbstract. p62/SQSTM1/Sequestosome-1 is an autophagic protein that serves a crucial role in cellular metabolism, proliferation and malignant growth. Notably, autophagy may influence the development and resistance to therapy of numerous types of human cancer. In the present pilot study, the immunohistochemical pattern of p62 was analyzed in a cohort of patients with isocitrate dehydrogenase (IDH)1/2 wild-type glioblastoma (GBM), in primary and recurrent samples, in order to verify the concordance or discordance between the primary and recurrent tumors. In addition, the association between p62, and patient outcome and O^6-methylguanine-DNA methyltransferase (MGMT) status was assessed. The results revealed p62 immunoexpression in the nucleus and cytoplasm of neoplastic elements in 45% of primary and 55% of recurrent cases of GBM. A discordant p62 immunoreactivity was detected in 35% of cases, with a variation either with positive or negative conversion of p62 status. Statistically, p62 expression and MGMT status exhibited a significant prognostic value by univariate analysis, whereas only MGMT promoter methylation status emerged as an independent prognostic factor by multivariate analysis. Finally, the most favorable prognosis was documented when the same GBM case was positively concordant for both p62 expression and MGMT methylated status. Since little data are available regarding the association between p62 expression and MGMT in GBM, further investigations may be required to determine if new targeted therapies may be addressed against autophagy-related proteins, such as p62.

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Key words: autophagy, p62/SQSTM1/Sequestosome-1, immunohistochemistry, O^6-methylguanine-DNA methyltransferase, glioblastoma

Introduction

Autophagy, already defined as an intracellular catabolic phenomenon, is considered to be involved in many pathophysiological processes, such as infection, autoimmune disease, neurodegenerative disorders, aging, cell death, and cancer (1-4). In the neoplastic field, it is well established that autophagy may exert a dual role, suppressing or contributing to tumorigenesis (5-10). During the autophagic process, many important autophagy-related proteins (ATGs) are involved either in its induction or the assembly, formation, and degradation of autophagosomes (11-14). Among the ATGs, a multifunctional protein considered autophagy adaptor is represented by p62, also named sequestosome 1 (SQSTM 1) (15,16); in particular, this protein may directly interact with microtubule-associated protein light chain 3 (LC3), and further, it may be specifically degraded by autophagy (15). Contrastingly, a defective autophagic phenomenon may produce a p62 upregulation in human tumors (17,18). Some reports have documented an evident p62 expression in pancreatic, hepatocellular, mammary, and oral squamous carcinomas, in which aggressive clinicopathological features and poor prognosis have been referred (16-20). In the light of these observations, it has been hypothesized that

p62 may promote the progression of cancer by repressing the apoptotic resistance and generating reactive oxygen species (ROS), thus, enhancing cell proliferation, tumorigenesis, and metastasis (20-23).

In the central nervous system (CNS), p62 has been mainly investigated in neurodegenerative disorders, such as Parkinson's and Alzheimer's diseases, as mitochondrial dysfunction is implicated in the pathogenesis of these disorders (24-26). Although previous reports have hypothesized the action of SQSTM1 as a regulator of mitochondrial function (27,28), additional studies on cell lines have raised some doubts concerning the exact role of p62 (29,30). Moreover, the function of p62 in the progression of glioma is not fully understood, even if in glioma stem-like cells, p62 should regulate invasion by modulating energy metabolism and affecting mitochondrial function (31,32). However, some data about p62 level in different glial neoplastic samples have been reported (15,33,34); remarkably, an increase in p62 expression has been progressively detected from low- to high-grade gliomas with prognostic value (15,33,34), although no correlation with isocitrate dehydrogenase (IDH) mutation status has been documented (15). In detail, a high p62 immunohistochemical expression has been reported in 34/81 primary high-grade gliomas and these patients had a lower mean of three years of overall survival (33). Moreover, a p62 immunoreactivity has been documented in 55/96 primary high grade (III and IV) gliomas with a positive correlation with overall survival and the proteins O^6-methylguanine-DNA methyltransferase (MGMT) and telomerase reverse transcriptase (TERT) promoter (34).

Glioblastoma (GBM) represents the most aggressive entity in CNS tumors, in which the IDH-wild-type constitutes over 90% of GBM, with a median overall survival (OS) ranging from 12 to 18 months and the 5-year survival of about 5% (35-37). The gold standard for the treatment of newly diagnosed GBM consists of maximal surgical exeresis, followed by concurrent radiotherapy/temozolomide (TMZ) and six-monthly cycles of adjuvant TMZ (38-40). If the tumor progresses after first-line therapy, a recurrent GBM (rGBM) occurs which makes the treatment a challenge, although many new drugs have been tested for their efficacy (40).

MGMT, a DNA repair protein, removes the alkylation at the O^6 position of guanine which is the most cytotoxic lesion induced by alkylating agent chemotherapy, such as nitrosoureas or temozolomide (TMZ) (8,9). However, some studies have compared the methylation status of the promoter for DNA repair protein MGMT in newly diagnosed tumors with matched recurrence samples after TMZ treatment (41-43). Low-level expression of MGMT protein impairs their ability to repair DNA. Hyper-methylation of MGMT gene promoter might result in silencing gene expression and further down-regulate protein concentrations (42,43). Few studies have analyzed if the MGMT methylation status of GBM might change during the disease course, with conflicting data and variable rates of change (5-40%) (41,42,44). Nevertheless, it is unclear whether this transition from methylated to unmethylated and *vice versa* in GBM recurrent tumors may be a result of TMZ treatment on MGMT status or due to the selection for a more drug-tolerant clone, or a mixture of both processes (43,45).

In the light of the above-mentioned well-known information concerning p62 immunoexpression and MGMT status, we have thought to perform as novelty an analysis regarding p62 immunohistochemical pattern in a cohort of IDH1/2 wild-type GBM, either in primary or recurrent, to verify if its expression is maintained or changed in relation to a potential association with relapse-free survival (RFS) or overall survival (OS). Additionally, the relationship between MGMT status and p62 immunoexpression in primary and corresponding recurrent IDH1/2 wild-type GBM has been analyzed, considering the rate of change of both parameters.

Materials and methods

Ethics approval. The study was conducted in accordance with Good Clinical Practice guidelines and the Declaration of Helsinki (1975, revised in 2013); its retrospective nature did not require any informed consent, although written informed consent had been obtained from each patient before surgical procedures. The clinical information had been retrieved from the patients' medical records and pathology reports. Patients' initials or other personal identifiers did not appear in any image. Finally, all samples were anonymized before histology and immunohistochemistry. Formal ethical approval was obtained from the Catania 1 Ethics Committee (Catania, Italy; protocol code: 166/2015/PO;17/12/2015).

Case selection. From the archives of the Department of Human Pathology of Adult and Evolutive Age (University of Messina, Messina, Italy) and the Department of Medical, Surgical Sciences, and Advanced Technologies 'G.F. Ingrassia' (University of Catania, Catania, Italy), 40 consecutive patients (26 men, 14 women; mean age, 55.85 years; range, 35-73 years) surgically treated for naïve IDH1/2 wild-type GBM were included in the present analysis. Initially, during routine pathology diagnostics, IDH1/2 status was analyzed by immunohistochemistry utilizing mouse monoclonal antibody IDH1 R132H (work dilution 1:50, clone H09, Dianova GmbH, catalogue n. 075874). Furtherly, the IDH1/2 wild type status on the same casuistry was verified utilizing IDH1/2 mutation detection kit for real-time PCR (EntroGen, product code IDH-RT38). For all cases, primary as well as recurrent neoplasms were available and histologically reviewed by two independent observers according to World Health Organization (WHO) 2016 criteria. Clinical characteristics of each patient, including age, sex, MGMT promoter methylation status assessed by quantitative polymerase chain reaction, disease-free interval, and overall survival were available from the medical records of our institution.

Immunohistochemistry. For immunohistochemical procedures, 5-micron thick sections obtained from corresponding tissue blocks were deparaffinized, then washed in descending alcohol scale, treated with 3% hydrogen peroxide for 10 min, washed again in deionized water for three times, and incubated with normal sheep serum to prevent unspecific adherence of serum proteins for 30 min at room temperature. Subsequently, sections were washed with deionized water and incubated for 30 min at 37˚C with commercially obtained against primary anti-human antisera mouse monoclonal anti-SQSTM1/p62

antibody (work dilution 1:200, clone 2C11, Abcam, catalogue n. ab 56416). Next, the sections were washed three times with PBS and incubated with a biotinylated goat anti-mouse IgG secondary antibody (1:300; Abcam, catalogue n. ab7064) for 20 min at room temperature, subsequently incubated with horseradish peroxidase-labeled secondary antibody for 30 min and developed with diaminobenzidine tetrahydrochloride and counterstained with hematoxylin using the ULTRA Staining system (Ventana Medical Systems). Negative controls were obtained by omitting the specific antisera and substituting PBS for the primary antibody. The assessment of p62 immunoreactivity was evaluated according to the intensity and percentage of positively stained cells, as elsewhere reported (2,4). The cytoplasmic and nuclear immunostaining intensity was rated as follows: 0, negative; 1, weak; and 2, strong. The percentage of positively stained cells was graded as follows: grade 0, 0-5%; grade 1, >5-25%; grade 2, >25-50%; grade 3, >50-75%; and grade 4, >75-100% for all antibodies. The immunoreactive score was calculated by adding the staining intensity score and the percentage score of positively stained cells (0-6). Tumors with an immunoreactive score of 0-3 were classified as negative, and those with a score of 4-6 were classified as positive. The immunohistochemical staining samples were independently scored by two pathologists (AI and GT), who were blinded to patient outcomes and other clinical findings, using a Zeiss Axioskop microscope (Carl Zeiss Microscopy GmbH) at 40x objective magnification. The interobserver agreement for p62 immunohistochemistry staining had a kappa value ranging from 0.73-0.80 (substantial agreement). One patient was considered p62 positive if primary or corresponding recurrent GBM showed protein expression.

MGMT pyrosequencing analysis. The MGMT analysis was done on the DNA extracted from paraffin-embedded tumor samples after bisulfite treatment and PCR amplification with primers specific for exon 1 of MGMT. Preliminarily, unmethylated cytosine residues were converted to uracil with bisulfite treatment of 500 ng DNA using the Epi Tect Bisulfite Kit (Qiagen) and the QiaCube automated purification system (Qiagen) according to the manufacturer's recommendation. The Therascreen MGMT Pyro Kit and the PyroMark Q24 system (Qiagen) were used to assess the methylation status of the MGMT gene promoter. Briefly, bisulfite-converted genomic DNA was amplified by PCR, the amplicons were immobilized on streptavidin beads, and single-stranded DNA was prepared, sequenced, and finally analyzed on the PyroMark Q24 System. The cut-off frequency for accepting methylation as positive was determined as elsewhere reported (46).

Statistical analysis. Statistical evaluation was performed using the SPSS version 13.0 software package (SPSS, Inc.). The association between p62 expression in GBM patients and clinicopathological features (age, sex, tumor site, MGMT status) was analyzed using the Chi-square (χ^2) or Fisher exact test. Cancer-specific survival analysis was performed by the Kaplan-Meier method, and for comparison of the survival curves, the Mantel-Cox log-rank test was used. A multivariate analysis (Cox regression model) was utilized to determine the independent effects of variables on overall survival. $P<0.05$ was considered to indicate a statistically significant difference.

Table I. Clinicopathological parameters in relation to p62 expression in 40 glioblastoma patients.

Parameter	No.	p62 expression (%)	P-value
Sex			NS
Male	26	18 (69.2)	
Female	14	9 (64.3)	
Tumour site			NS
Frontal	10	7 (70)	
Parietal	9	4 (44.4)	
Fronto-parietal	5	3 (60)	
Temporal	16	13 (81.3)	
MGMT promoter methylation status			<0.001
Methylated	18	18 (100)	
Unmethylated	22	9 (40.9)	

NS, not significant; MGMT, O^6-methylguanine-DNA methyltransferase.

Table II. Sub-grouping for p62 immunoreactivity.

Number of cases	Primary GBM	Recurrent GBM
13[a]	p62 +ve	p62 +ve
13[b]	p62 -ve	p62 -ve
14[c]	5 p62 +ve	p62 -ve
	9 p62 -ve	p62 +ve

[a]Positive concordant group; [b]negative concordant group; [c]discordant groups. GBM, glioblastoma.

Results

Clinicopathological parameters, as well as immunohistochemical data on p62 expression, are summarized in Table I. In our cohort, the p62 immunoexpression was found in the nucleus and cytoplasm of neoplastic elements in 18/40 (45%) primary (Fig. 1A and B) and 22/40 (55%) recurrent GBM (Fig. 2). By contrast, 22 primary GBM, as well as 18 recurrent GBM were consistently unstained (Fig. 3). Moreover, healthy normal nervous tissue neighboring GBM exhibited a constant p62 negative immunostaining (Fig. 4).

Table II showed the concordance, either negative or positive, respectively in 13/40 (32.5%) and 13/40 (32.5%); moreover, a discordant p62 immunoreactivity was found in 14/40 (35%), of which in 5/40 (12.5%) a change from positive to negative was encountered, while in 9/40 (22.5%) a variation from negative to positive was found (Table II). In particular, the additional Table III offered p62 detailed ID score for all cases analyzed, either primary or recurrent. Therefore, analyzing the p62 expression in primary and recurrent GBMs, three subgroups may be identified: Positive concordant, negative concordant and discordant; the difference among them ($\chi^2=6.814$) was

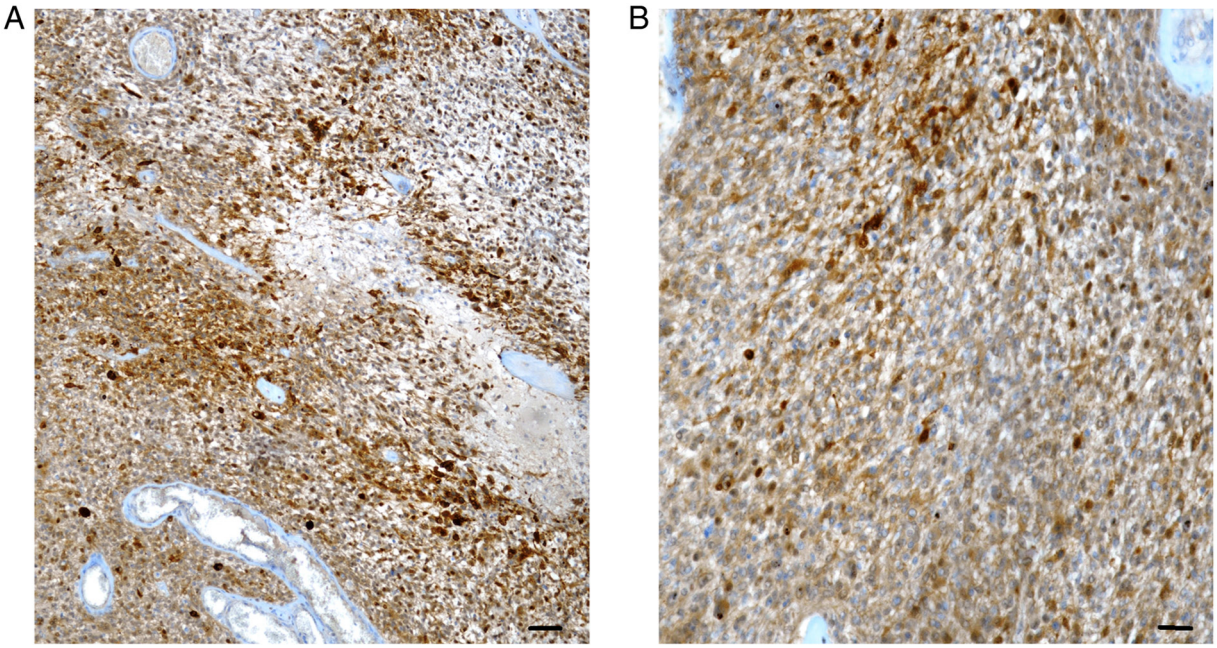

Figure 1. An evident strong and diffuse nuclear/cytoplasmic immunoreactivity was documented in primary glioblastoma, either at (A) low (magnification, x120) or (B) high (magnification, x360) magnification (p62 antiserum, Mayer's nuclear counterstain). Scale bar, 50 μm.

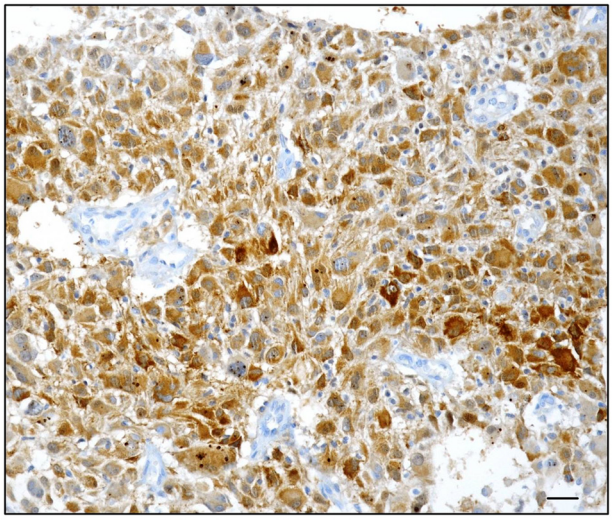

Figure 2. Diffuse homogeneous p62 immunopositivity was found in recurrent glioblastoma, (magnification, x480; Mayer's nuclear counterstain). Scale bar, 50 μm.

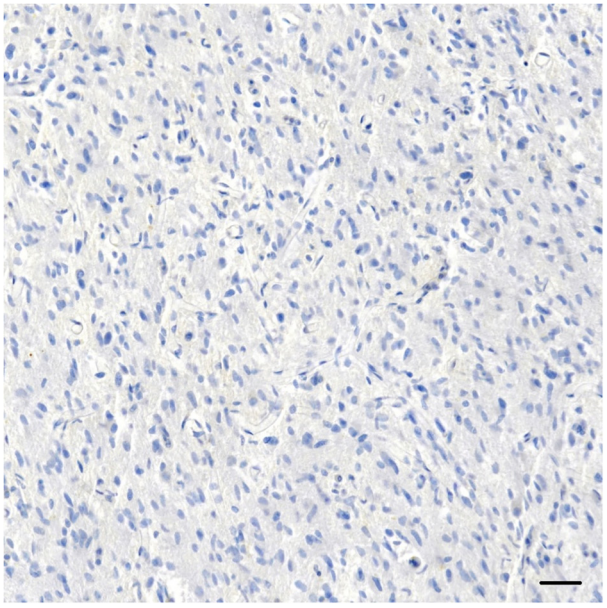

Figure 3. An absent uniform p62 immunoreactivity was evident in some glioblastoma (magnification, x200; Mayer's nuclear counterstain). Scale bar, 50 μm.

statistically significant (P=0.033). It merged that the most favorable prognosis was achieved when the same GBM case was positively concordant for both parameters, in comparison to other groups (χ^2=14.538), exhibiting a significant statistical value (P=0.001), as shown in survival curves performed by the Kaplan-Mayer method (Fig. 5).

Concerning MGMT status 18/40 cases showed a methylated profile (Fig. 6). In a univariate analysis of GBM patients, MGMT promoter methylation status (χ^2=14.517) and p62 expression (χ^2=6.590) showed a significant P-value (Table IV). By multivariate survival analysis, only MGMT promoter methylation status emerged as an independent prognostic parameter (Table V).

Discussion

In the present pilot study, we have analyzed the immunohistochemical expression of p62 in a cohort of GBM, considering each patient as positive when this autophagic protein was indifferently revealed in primary and/or corresponding recurrent GBM samples. We have found a p62 immunoreaction in the nucleus and cytoplasm of neoplastic elements in 45% of GMB primary and 55% recurrent cases. However, a variable rate of p62 immunostaining has been

Table III. Detailed information concerning p62 immunoreactive score either in primary or recurrent GBM.

Case nr.	Age, years	Sex	Location	ID score p62_ primary	ID scorep62_ recurrence
1	61	F	Temporal	0	0
2	62	M	Parietal	1	1
3	51	F	Temporal	5	2
4	64	F	Temporal	6	1
5	70	F	Frontal	0	0
6	54	M	Temporal	4	4
7	39	M	Frontal	0	0
8	53	M	Fronto-parietal	6	6
9	55	F	Temporal	5	4
10	53	M	Temporal	6	5
11	62	M	Fronto-parietal	2	2
12	35	M	Frontal	5	5
13	62	M	Parietal	4	0
14	61	M	Temporal	6	5
15	63	M	Temporal	4	5
16	49	F	Frontal	1	4
17	49	M	Temporal	5	5
18	52	M	Parietal	2	5
19	49	M	Frontal	1	4
20	49	M	Temporal	0	5
21	57	F	Temporal	0	0
22	70	M	Parietal	0	0
23	73	M	Temporal	1	6
24	47	M	Fronto-parietal	4	4
25	57	M	Parietal	5	6
26	37	F	Temporal	4	0
27	70	F	Temporal	0	0
28	50	M	Frontal	6	6
29	65	M	Parietal	1	1
30	65	M	Temporal	2	6
31	66	F	Frontal	5	5
32	59	M	Temporal	0	5
33	66	F	Parietal	0	4
34	45	M	Frontal	1	1
35	52	F	Parietal	0	0
36	41	M	Frontal	6	6
37	52	M	Parietal	1	0
38	65	M	Fronto-parietal	0	0
39	48	F	Fronto-parietal	0	5
40	56	F	Frontal	4	0

Tumors with an immunoreactive score of 0-3 were classified as negative while those with a score of 4-6 were considered positive. F, female; M, male.

Table IV. Prognostic parameters examined in glioblastoma cases: A univariate analysis of cancer-specific mortality by Mantel-Cox log-rank test.

Variable	χ^2	df	P-value
MGMT methylation status	14.517	1	<0.001
p62 expression	6.590	1	0.010

df, degrees of freedom; MGMT, O^6-methylguanine-DNA methyltransferase.

Table V. Multivariate survival analysis by Cox regression model in glioblastoma patients.

Variable	β	SE	Exp(β) RR	CI 95% Exp(β)	P-value
MGMT methylation status	0.612	0.174	1.843	1.311-2.592	<0.001
p62 expression	-	-	-	-	0.822

β, regression coefficient; SE, standard error Exp(β) RR, ratio of risk; CI, 95% confidence interval with lowest and highest values.

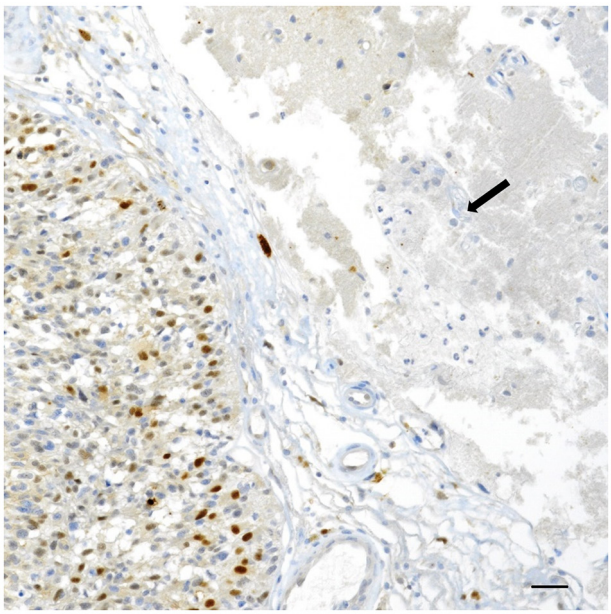

Figure 4. Note the negative p62 immunostaining in the neighboring healthy nervous tissue (black arrow) in comparison to glioblastoma in the left corner (magnification, x200; Mayer's nuclear counterstain). Scale bar, 50 μm. 3. An absent uniform p62 immunoreactivity was evident in some glioblastoma (magnification, x200; Mayer's nuclear counterstain). Scale bar, 50 μm.

elsewhere reported in primary high-grade gliomas (33,34); specifically, the reported positive percentage ranged from 42 to 57% (33,34). These values are greatly superimposable with ours in primary and recurrent GBM. In addition, our data confirm that an increase in p62 protein was detected in about 50% of GBM cases analyzed, with a concordant rate of 65% between primary and recurrent GBM. Interestingly, a discordant p62 immunoreactivity was found in 35% of GBM

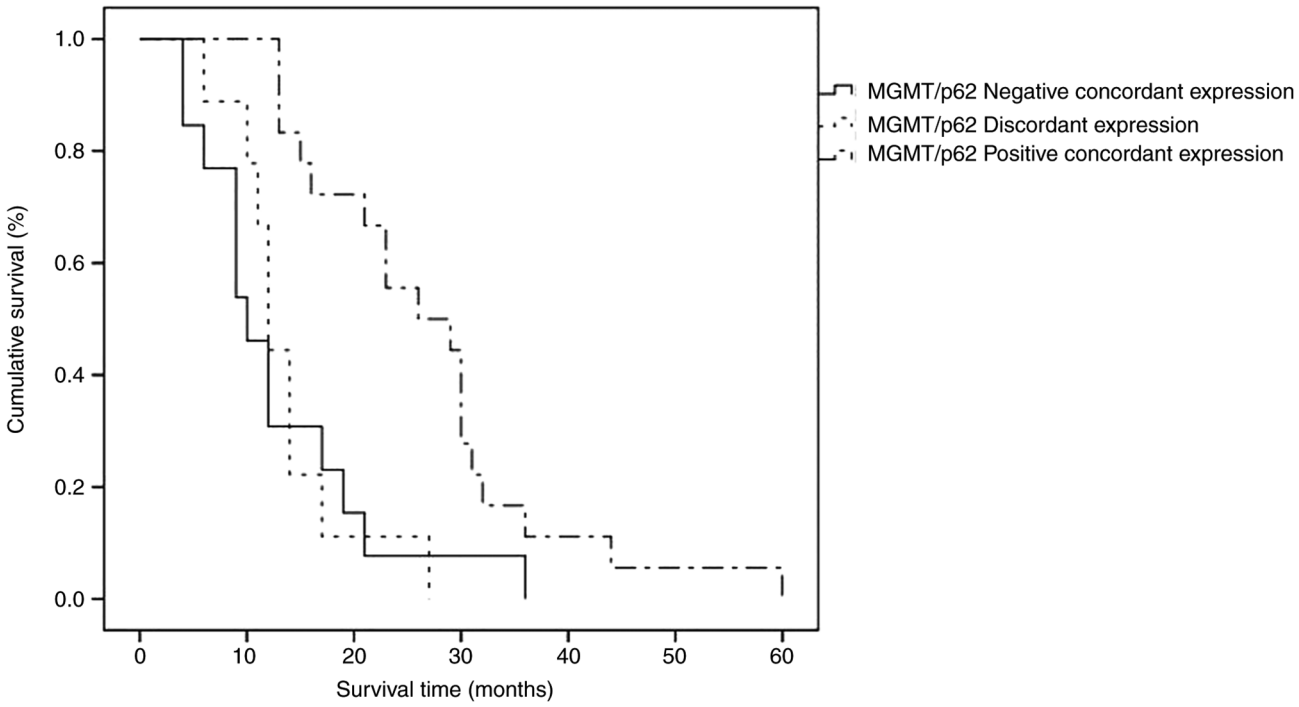

Figure 5. Survival curves in relation to concordant/discordant status of MGMT/p62 expression in primary and corresponding recurrent glioblastoma. MGMT, O^6-methylguanine-DNA methyltransferase.

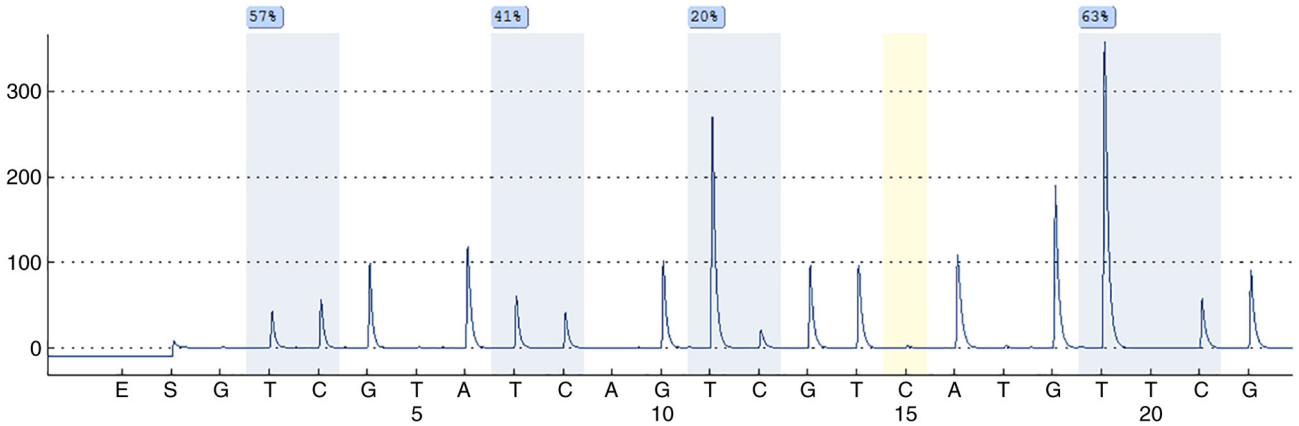

Figure 6. Pyrogram of a glioblastoma O^6-methylguanine-DNA methyltransferase methylated case.

cases, although a variation from negative to positive and *vice versa* has been documented. The occurrence of changes in biomarker expression in tumors represents biological evidence frequently observed in oncology. As largely documented in the literature, a lack of concordance of oncogene expression (i.e., HER2) has been reported between primary and metastatic/recurrent neoplasias, such as breast and gastric cancer (47-49); therefore, a similar phenomenon may also be suggested in brain gliomas. However, to explain the documented change in biomarker expression, many different mechanisms have been hypothesized such as intratumoral heterogeneity, clone selection promoted by cytotoxic treatments, and lastly analytical bias (47-49).

A progressive p62 enhancement moving from the WHO grade II to grade IV, as previously elsewhere suggested (15,33). Moreover, p62 overexpression has been reported also in glioma cell lines and no difference in p62 expression between IDH wild-type or IDH mutated groups was reported, suggesting that p62 function may be considered independent of IDH status (15,33). Consequently, it can be argued that p62 overexpression stimulates the classical autophagic pathway, allowing GBM cell survival by antagonizing apoptosis and producing drug resistance to proteasome inhibitors (17,50,51). Alternatively, an accumulation of the autophagy substrate p62 may reveal a defective process that cannot degrade its substrates. Therefore, p62 may act as a tumor promoter in glioma cells not only by the regulation of autophagy but also by interfering with proliferation, migration, and Temozolomide resistance (15).

The 2016 classification by WHO of brain tumors introduced new molecular markers in high-grade gliomas, such as MGMT methylation, IDH1, TP53, and TERT promoter

mutation (52); this approach may represent a crucial point in the neoplastic strategy treatment, predicting the sensitivity of gliomas to chemotherapy as well as the prognosis (53-56). In the present paper, we have combined the p62 expression and MGMT promoter methylation status to evaluate if an association between these two parameters may be appreciable; in detail, in relation to this point, three groups may be identified: negative concordant, positive concordant and discordant. Taking into consideration the suggestion that MGMT promoter methylation presence has been considered as an independent favorable prognostic factor in GBM, we have documented the achievement of the most favorable prognosis when the same GBM case was positively concordant for both p62 expression and MGMT methylated status. Interestingly, this association is further emphasized by the comparative analysis of primary and corresponding recurrent GBM in relation to MGMT methylation. Therefore, a significant association between these latter two parameters should be hypothesized, similarly to that elsewhere reported (34). On the other hand, the univariate analysis allowed us to identify MGMT promoter methylation status as well as p62 expression as significant prognostic factors able to define GBM long survivors, although only MGMT methylation emerged as an independent marker in multivariate analysis. These data confirm recent findings that have demonstrated a worse prognostic behavior in GBM patients with high levels of autophagy-related genes and MGMT promoter unmethylated (57). However, autophagy can have a tumor suppressor function in GBM destroying damaging unfolded proteins, oncogenic protein substrates, and injured organelles (58,59). Recently, it has been reported that elevated levels of ATGs were linked to better survival in glioma patients (60-62). In particular, higher AKT and mTOR hyperphosphorylation has been reported in high-grade gliomas in comparison to low-grade ones (63,64). It has been suggested that mTOR signaling pathway activation is associated with autophagy inhibition, supporting the glioma stem cell proliferation, tumor infiltration, and therapeutic resistance (65,66).

Although the relationship between autophagy and programmed cell death is not fully elucidated, we may hypothesize that the capability to repair DNA damage should be reduced by a methylated MGMT status, and therefore, autophagy and apoptosis may interact with each other through several pathways. However, the coexistence observed by us of p62 expression and MGMT profile in GBM needs to be analyzed further in their putative prognostic role, since only a few data are available on the association between autophagy and other synchronized mutations and therefore, in the future, an extensive study on a larger cohort should be carried out as the next step.

Acknowledgements

The authors would like to thank Professor Sandra De Dominici for her assistance in reviewing the English style and grammar of the manuscript.

Funding

This research was funded by grants from the Italian Minister of Research and University (FFABR ANVUR 2021).

Availability of data and materials

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

Authors' contributions

AI and GT designed the project and wrote the paper. CP, GB, AG, GMVB and PV contributed to data collection and analysis. RC and GG analyzed data and critically reviewed/edited the manuscript. AI and GT confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Catania 1 Ethics Committee (Catania, Italy; protocol code: 166/2015/PO;17/12/2015).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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