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Melanocortin Receptor-4 Gene Polymorphisms in Glioblastoma Patients Treated with Concomitant Radio-Chemotherapy

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Abstract Melanocortins are peptides with well-recognized antiinflammatory and neuroprotective activity. No data are currently available on *melanocortin receptor-4* (*MC4R*) gene polymorphisms and tumors, including glioblastomas (GBMs), or their relationship with radiotherapy or chemotherapy. The aim of this study was to evaluate the possible predictive/prognostic role of the *MC4R* SNPs on GBM patients. Fifty-five patients with a proven diagnosis of GBM, treated with radiotherapy and temozolomide, were consecutively enrolled. *MC4R* gene SNPs (rs17782313, rs489693, rs8087522, rs17700633) were analyzed by a validated TaqMan® SNP genotyping assays. Univariate and multivariate analyses were performed. A $P < 0.0125$ (Bonferroni's correction) was considered significant (Clinicaltrial.gov identifier NCT02458508). The median progression-free survival (PFS) and median overall survival (OS) of these patients were 9.54 (95% CI 5.4–14.3) months and 24.9 (95% CI 17.8–34.6) months, respectively. The MC4R rs489693 AA genotype was significantly associated with a shorter PFS and OS. Indeed, with regard to PFS, patients harboring the rs489693 AA genotype had a median PFS of 2.99 months whereas patients with AC/CC genotypes had a median PFS of 10.82 months ($P = 0.009$). Interestingly, the rs489693 AA patients also had a lower median OS as compared with the median OS of the AC/CC genotypes (10.75 vs. 29.5 months, respectively, $P = 0.0001$). This study suggests that the *MC4R* rs489693 AA genotype is significantly associated with a shorter PFS and OS in patients treated with radiotherapy and temozolomide. These findings represent a relevant effort to identify novel clinical markers for RT–CT therapy in GBM to be validated in future pharmacogenetic clinical trials.

Keywords Glioblastoma · Melanocortin receptor-4 · Polymorphism · Radiotherapy · Temozolomide

Introduction

Glioblastoma (GBM) represents the most frequent malignant primary brain tumor and, despite the latest advances recorded

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over the past decade, remains a lethal disease with a dismal prognosis [1]. The treatment of glioblastomas remains difficult in that no contemporary treatments are curative. The current standard of care for newly diagnosed GBM was established in 2005, after the conclusion of the study leaded by Stupp [2] and nowadays, upfront concomitant radiotherapy–chemotherapy (RT–CT) with temozolomide (TMZ) represents the gold standard of care. Unfortunately, the recurrence rate after concomitant RT-CT and the overall survival (OS) are still unsatisfactory; indeed, 90% of patients experienced a disease relapse and the median OS in clinical trial population was 15–18 months [3]. Despite important advances in the characterization of genomic and microenvironment alterations in glioblastoma, targeted agents have shown minimal efficacy in clinical trials to date, and patient survival remains poor. Indeed, large placebo-controlled randomized phase III trials aimed at targeting the angiogenic phenotype of glioblastoma using bevacizumab (an antivascular endothelial growth factor, VEGF) or cilengitide (an integrin inhibitor) failed to show an improvement in overall survival compared with the current standard of care [4].

Melanocortins are peptides with well-recognized antiinflammatory and neuroprotective activity [5, 6]. Of the five known melanocortin receptors, only subtype 4 is present in astrocytes [7]. Melanocortin receptor-4 (MC4R) [8] has been shown to mediate melanocortin effects on energy homeostasis [9], inflammation [10], neuroprotection, and neurogenesis [6, 8, 11, 12] and, recently, to modulate astrocyte functions [7]. Recently, in experimental brain ischemia, treatment with melanocortins acting at MC4Rs induced neural stem/progenitor cell proliferation by triggering the canonical wingless-type MMTV integration site (Wnt)-3A/β-catenin and Sonic hedgehog (Shh) signaling pathway [13]. Moreover, Caruso and colleagues demonstrated that MC4R activation by α-melanocyte-stimulating hormone (α-MSH) protects astrocytes from apoptosis by decreasing caspase-3 activity and stimulates proliferative effects in 7-day-old cultured astrocytes [7].

MC4R gene polymorphisms have emerged as new and promising biomarkers for the prediction of weight gain in patients treated with antipsychotic drugs [14]. Several independent genome-wide association studies identified the polymorphism *MC4R* rs17782313 to be linked to increased body weight and obesity [15], having shown an effect on body mass index (BMI) in different populations [16, 17]. Moreover, a direct role in the interaction between *fat mass- and obesity-associated* (FTO) and *MC4R* gene polymorphisms in breast cancer development has been recently demonstrated [18]. On the other hand, the *MC4R* rs17782313 polymorphism was not related to endometrial cancer risk [19] although overweight and obesity are strongly associated with this type of cancer. Recently, no evidences were found supporting that individual variants in *MC4R* gene are associated with risk of colorectal cancer [20]. No data are currently available on *MC4R* gene polymorphisms and GBMs or their relationship with the success or failure to radio- or chemotherapy. The aim of this study was to evaluate the possible prognostic/predictive role of the *MC4R* SNPs on GBM therapy.

Patients and Methods

This was an exploratory, multicenter retrospective pharmacogenetic study. Fifty-five patients with age ≥18 years and diagnosis of GBM were recruited, consecutively, and they were assessed for the present pharmacogenetic study. The patients evaluated in this study were treated with post-operative concomitant RT and CT. RT was delivered to surgical bed plus residual disease with 2 cm of margin; the total dose delivered was 60 Gy in 30 fractions. CT was represented by TMZ that was administered at the dose of 75 mg/m^2/day during the RT time and of 150–200 mg/m^2/day for 5 days every 28 days during 6–12 cycles, until disease progression or toxicity occurrence. Disease relapse was assessed 1 month after the end of concomitant RT–CT with a contrast magnetic resonance imaging (MRI) and every 3 months for the first 3 years, then every 4 months (if a pseudoprogression was suspected, a new MRI was performed after 6 weeks). Macdonald criteria were used to define disease control [21]. Each patient entering the study signed the informed consent. The protocol was approved by the Comitato Etico di Area Vasta Nord Ovest (CEAVNO), Pisa, Italy (CEAVNO prot. n. 17013; clinicaltrial.gov identifier NCT02458508) and by the ethic committees of all participating centers.

SNP Selection

The *MC4R* gene SNPs (rs17782313, rs489693, rs8087522, rs17700633) included in our study (supplementary Table 1) were selected on the basis of four main considerations: (i) these SNPs have been described to be associated with human diseases such as obesity and diabetes or with antipsychotic-induced weight gain but not with cancer; (ii) only the rs17782313 has been significantly associated with risk of breast cancer [18] but not in the endometrial [19] and colorectal cancer [20]; (iii) thus far, no genetic analysis has been reported on the chosen *MC4R* SNPs and GBM; and (iv) although for the chosen SNPs the phenotypic effects are still undefined (i.e., the modulation of the gene expression or activity of the receptor), we decided to include them in our pilot research because of the possibility that some genotypes or haplotypes could determine a statistical effect on patient's survival.

Genotyping Analyses

Blood samples (3 ml) were collected in ethylenediaminetetraacetic acid (EDTA) tubes and stored at −80 °C. Germline DNA extraction was performed using QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA). Allelic discrimination of genes was performed using an ABI PRISM 7900 SDS

(Applied Biosystems, Carlsbad, CA, USA) and with validated TaqMan® SNP genotyping assays (i.e., C_32667060_10, rs17782313; C_3058718_10, rs489693; C_29004626_10, rs8087522; C_32666984_10, rs17700633; Applied Biosystems). Polymerase chain reaction (PCR) reactions were carried out according to the manufacturer's protocol. Genotyping was not performed until an adequate number of events (>80% on study population) was reported in terms of progression-free survival (PFS).

Statistical Analysis

The statistical analysis was performed by V. Simeon. All polymorphisms were analyzed for deviation from the Hardy–Weinberg Equilibrium (HWE) through comparison between observed allelic distributions with those expected from the HWE by on χ^2 test. Linkage disequilibrium (LD) between markers in *MC4R* (n = 4) was analyzed using Haploview software package [22]. The difference in PFS or OS between genotype profiles was assessed with the log-rank test and the Kaplan–Meier method to evaluate survival curves. A Cox proportional hazards model, with the genotype profiles of each individual polymorphism and the clinical and pathological patient characteristics individually correlated with the PFS or OS, was used to calculate the adjusted hazards ratio (HR) and the 95% confidence interval (95% CI). In these analyses, we used Bonferroni's correction and the P value <0.0125 (0.05/4 SNPs = 0.0125) was accepted as statistically significant. The Kaplan–Meier and Cox proportional hazard analyses were performed using the STATA package version 11.0 (StataCorp). Where an association was found to be significant on univariate analyses, multivariate analysis was performed. PFS was defined as the period of time from the beginning of the RT–CT treatment to the first observation of disease progression as above described, or death from any cause. OS was calculated from the date of the diagnosis to the date of death/lost to follow-up. Due to the small sample size in OS data, multivariate analysis was restricted to only three variables. Decision on best model was performed calculating Bayesian Information Criterion (BIC) [23].

PHASE software v2.1 [24] was used to perform haplotype analysis for *MC4R* regions separately. Only common haplotypes with a frequency ≥5% were included in Cox survival analysis. The most common haplotype was used as the reference and were provided hazard ratios (HR) for a given haplotype relative to reference haplotype.

Results

Fifty-five patients with diagnosis of GBM were enrolled into the study and genotypized for *MC4R* SNPs. Details about genotypes and allele frequencies of all SNPs in the entire population are reported in supplementary Table 1; all the SNPs were in HWE. The clinical characteristics of the 55 patients are summarized in Table 1. All patients were clinically assessable for PFS and OS. The PFS of these patients was 9.54 months (95% CI 5.4–14.3), whereas the OS resulted 24.9 months (95% CI 17.8–34.6). The hematological toxicity (i.e., leucopenia and thrombocytopenia) was recorded during the RT–CT treatment and reported in supplementary Table 2.

The univariate Cox regression analysis showed the role of the age at diagnosis (HR = 1.03; P = 0.035) and of the frontal localization (HR = 0.46; P = 0.028) of the disease in determining the PFS of this group of patients (supplementary Table 3). The same univariate analysis confirmed the role of the frontal lobe site (HR = 0.38; P = 0.026) of the disease in determining the OS with the addition of performance status (HR = 0.98; P = 0.03) and the administered lines of chemotherapy (HR = 0.6; P = 0.031) (supplementary Table 4).

Table 1 Characteristics of patients

Characteristics	
Sex, *n* (%)	
Male	27 (49.1)
Female	28 (50.9)
Weight	
Mean ± SD	73.3 ± 12.9
Median (range)	72.5 (52–103)
Age at diagnosis	
Mean ± SD	56.6 ± 12.4
Median (range)	58 (24–80)
Body mass index (BMI)	
Mean ± SD	25.1 ± 3.97
Median (range)	25 (21.6–27.8)
Previous radiotherapy, *n* (%)	
No	36 (65.45)
Yes	16 (29.09)
Unknown	3 (5.45)
Lines of chemotherapy, *n* (%)	
1	25 (45.45)
2	16 (29.09)
3	10 (18.18)
4	3 (5.45)
Unknown	1 (1.82)
Frontal lobe site, *n* (%)	
No	34 (61.82)
Yes	17 (30.91)
Unknown	4 (7.27)
Performance status	
Mean ± SD	78.9 ± 19.7
Median (range)	80 (30–100)

A statistically significant association with PFS in univariate Cox regression analysis, either under an additive, dominant, or recessive model (as illustrated in Table 2), was found with *MC4R* rs489693 that reaches the significance threshold in both the additive (P = 0.012) and dominant models (P = 0.009). Indeed, the PFS values of *MC4R* rs489693 AA, CA, and CC patients were 2.99 (95% CI 2.3–4.5), 9.77 (95% CI 5.1–22.7), and 10.82 (95% CI 5.4–17.8) months, respectively (Fig. 1a), whereas the PFS of CC/CA genotypes was 10.82 (95% CI 5.7–15.8) months vs. 2.99 (95% CI 2.3–4.5) months of the harboring AA patients (Fig. 1b). Interestingly, the same SNP maintained the same statistically significant association with OS (n = 53) in univariate Cox regression analysis, either under an additive (P = 0.0001) or a dominant (P = 0.0001) model as shown in Table 3. Indeed, the OS values of *MC4R* rs489693 AA, CA, and CC patients were 10.75 (95% CI 7.4–12.5), 25.75 (95% CI 13.5–57.3), and 34.1 (95% CI 17.7–73.2) months, respectively (Fig. 1c), whereas the OS of CC/CA genotypes was 29.5 (95% CI 22.6–40.1) months vs. 10.75 (95% CI 7.4–12.5) months of the harboring AA patients (Fig. 1d). Furthermore, the haplotype analysis of the *MC4R* genetic region (supplementary Fig. 1a) did not reveal any significant association in univariate Cox regression model and Kaplan–Meier PFS (n = 55; supplementary Fig. 2b) and OS (n = 53; supplementary Fig. 1c) curves.

Notably, the multivariate analyses adjusted for the age at the diagnosis, frontal lobe localization, and lines of chemotherapy revealed that the *MC4R* rs489693 AA genotype was the most powerful single factor associated with the PFS (HR = 3.45 and P = 0.018 for the additive model; HR = 3.14 and P = 0.021 for the dominant model), as described in Table 4. The best models in multivariate analyses with OS data, selected with BIC value (supplementary Table 5), were both additive and dominant models of *MC4R* rs489693 (HR = 6.1 and P = 0.004 for the additive model; HR = 4.9 and P = 0.006 for the dominant model) adjusted for performance status and frontal lobe localization (Table 5). The one with the dominant model shows the lowest BIC value with positive to strong evidence to be preferred against the others.

No association between *MC4R* SNPs and weight of patients at the diagnosis was found (data not shown), as well as between *MC4R* SNPs and localization of the disease or hematological toxicities (data not shown). Although analyzed in a small subset of patients (n = 27) with available tumor tissues, as expected, the tumor O^6-methylguanine-DNA methyltransferase (MGMT) methylation significantly increase the PFS (15.8 vs. 5.13 months, P = 0.02; n = 27; supplementary Fig. 2a) and, even though it did not reach a statistical significance, also the OS (25.9 vs. 17.8 months, P = 0.25; n = 27; supplementary Fig. 2b).

Discussion

The present study describes, for the first time, the association between *MC4R* polymorphisms and survival of GBM patients under RT–CT regimen that consists of TMZ and concomitant RT (60 Gy). In this regard, the individual genetic traits of patients may have a role in the response to chemotherapy or to radio-therapeutic strategies in GBM. Indeed, the TMZ-treated GBM patients harboring the T allele of the *MGMT* promoter SNP rs16906252 have shown a better survival, independently of the tumor methylation status [25]. However, with the exception of the establish role of MGMT methylation [26], there are currently no validated genetic biomarkers to predict or to monitor favorable clinical response or resistance to concomitant radio- and TMZ therapies [27]. Therefore,

Table 2 Association between each *MC4R* polymorphism and PFS (univariate Cox regression model)

ID	Additive model					Dominant model					Recessive model				
	Genotype	*N*	HR	*P*	95% CI	Genotype	*N*	HR	*P*	95% CI	Genotype	*N*	HR	*P*	95% CI
rs17782313	TT	30	1	–	–	TT/TC	53	1	–	–	TT	30	1	–	–
	TC	23	1.33	0.35	0.73–2.42						TC/CC	25	1.4	0.26	0.78–2.52
	CC	2	3.31	0.11	0.76–14.4	CC	2	2.92	0.14	0.69–12.4					
rs489693	CC	30	1	–	–	CC/CA	49	1	–	–	CC	30	1	–	–
	CA	19	0.97	0.93	0.51–1.85						CA/AA	25	1.2	0.53	0.67–2.17
	AA	6	3.26	0.012	1.29–8.22	AA	6	3.29	0.009	1.34–8.04					
rs8087522	GG	32	1	–	–	GG/GA	52	1	–	–	GG	32	1	–	–
	GA	20	0.8	0.5	0.43–1.5						GA/AA	23	0.78	0.44	0.43–1.44
	AA	3	0.66	0.57	0.16–2.8	AA	3	0.72	0.66	0.18–3					
rs17700633	GG	35	1	–	–	GG/GA	54	1	–	–	GG	35	1	–	–
	GA	19	1.82	0.055	0.98–3.38						GA/AA	20	1.54	0.16	0.84–2.83
	AA	1	[a]	1	[a]	AA	1	[a]	1	[a]					

A P value <0.0125 was defined as statistically significant (Bonferroni's correction)

[a] The value was not determined because of the occurrence of only one AA case

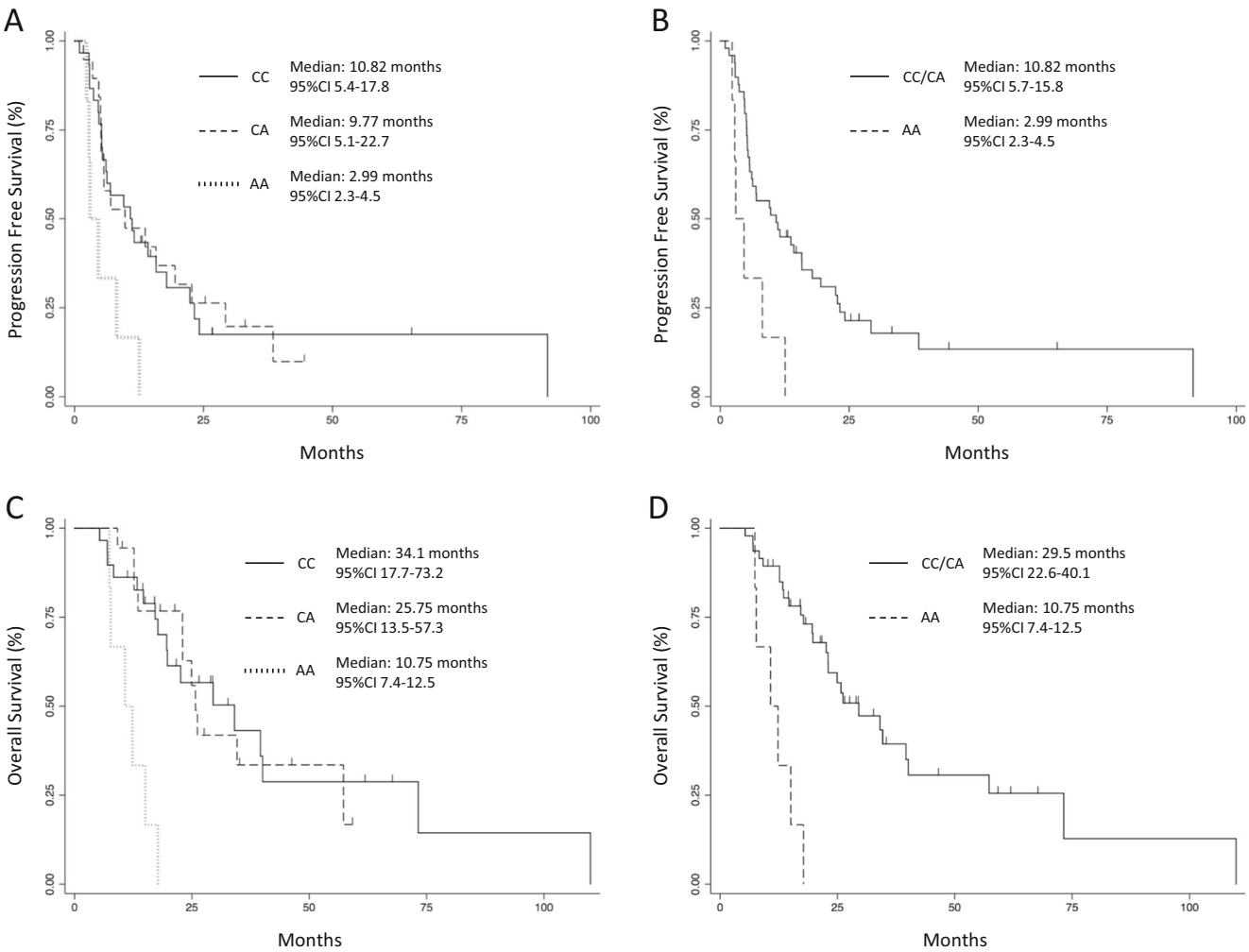

Fig. 1 Kaplan–Meier curves for progression-free survival (PFS) of patients harboring **a**) *MC4R* rs489693 CC, CA, and AA genotypes and **b**) *MC4R* rs489693 CC/CA and AA genotypes. Kaplan–Meier curves for overall survival (OS) of patients harboring **c**) *MC4R* rs489693 CC, CA, and AA genotypes and **d**) *MC4R* rs489693 CC/CA and AA genotypes. *95% CI* 95% confidence interval

Table 3 Association between each *MC4R* polymorphism and OS (univariate Cox regression model)

ID	Additive model					Dominant model					Recessive model				
	Genotype	*N*	HR	*P*	95% CI	Genotype	*N*	HR	*P*	95% CI	Genotype	*N*	HR	*P*	95% CI
rs17782313	TT	29	1	–	–	TT/TC	51	1	–	–	TT	29	1	–	–
	TC	22	1.94	0.075	0.94–3.9						TC/CC	24	2.08	0.041	1.03–4.2
	CC	2	6.1	0.022	1.3–28.7	CC	2	4.47	0.049	1.01–19.9					
rs489693	CC	29	1	–	–	CC/CA	47	1	–	–	CC	29	1	–	–
	CA	18	1.08	0.855	0.5–2.4						CA/AA	24	1.5	0.24	0.75–3.04
	AA	6	7.02	0.0001	2.44–20.2	AA	6	6.8	0.0001	2.5–18.6					
rs8087522	GG	30	1	–	–	GG/GA	50	1	–	–	GG	30	1	–	–
	GA	20	0.79	0.53	0.4–1.65						GA/AA	23	0.84	0.61	0.42–1.7
	AA	3	1.23	0.78	0.3–5.3	AA	3	1.35	0.69	0.32–5.7					
rs17700633	GG	34	1	–	–	GG/GA	52	1	–	–	GG	34	1	–	–
	GA	18	1.44	0.36	0.66–3.15						GA/AA	19	1.25	0.58	0.6–2.7
	AA	1	[a]	1	[a]	AA	1	[a]	1	[a]					

A *P* value <0.0125 was defined as statistically significant (Bonferroni's correction)

[a] The value was not determined because of the occurrence of only one AA case

Table 4 Multivariate Cox regression model of PFS

	HR	*P*	95% CI
Age at diagnosis	1.01	0.311	0.98–1.04
Lines of chemotherapy	0.82	0.311	0.55–1.2
Frontal lobe site	0.46	0.058	0.2–1.03
rs489693_additive			
CC	1	–	–
CA	1.25	0.558	0.6–2.6
AA	3.45	0.018	1.23–9.6
Age at diagnosis	1.01	0.291	0.98–1.04
Lines of chemotherapy	0.81	0.297	0.55–1.2
Frontal lobe site	0.50	0.07	0.24–1.06
rs489693_dominant			
CC/CA	1		
AA	3.14	0.021	1.2–8.3

Lines of chemotherapy is represented as an ordinal variable

HR hazard ratio, *CI* confidence interval

efforts to identify new genetic biomarkers for non-invasive detection of treatment success are an important component of GBM research to improve the overall survival and quality of life of GBM patients. In this perspective, a recent study has associated the *suppressor of Lin-12-like* (*Caenorhabditis elegans*) (*SEL1L*) rs12435998 C allele with a prolonged OS (18 vs. 13 months, $P = 0.011$) and a better response to TMZ-based radio–chemotherapy (i.e., Stupp's protocol) in 55 GBM patients [28]. Moreover, Di Stefano and colleagues have demonstrated that the *VEGF-A* rs2010963 CC genotype is associated with longer PFS and higher risk of vascular events in patients with recurrent GBM if treated with bevacizumab, but not when treated with the TMZ [29]. Here, we report the impact of commonly reported *MC4R* sequence variants, usually described in the gain of weight during antipsychotic therapy [30–33], in relation to clinical survival following RT–CT concomitant treatment. In particular, we decided to test the hypothesis that, given the association between MC4R with prevention of astrocyte apoptosis [7], induction of neural stem/progenitor cell proliferation in brain hypoxia [11], neuroprotection, and antiinflammatory activity [6], the *MC4R* SNPs could have a possible direct role in the modulation of the cytotoxic, and thus therapeutic, effects of radiotherapy and TMZ on GBM or they could influence the prognosis of the disease through their effect on patient's tumors.

Table 5 Multivariate Cox regression model of OS

	HR	*P*	95% CI
Performance status	0.99	0.39	0.97–1.01
Frontal lobe site	0.42	0.052	0.17–1.01
rs489693_dominant			
CC/CA	1		
AA	4.9	0.006	1.6–15.8
Performance status	0.99	0.37	0.97–1.01
Frontal lobe site	0.38	0.035	0.15–0.94
rs489693_additive			
CC	1		
CA	1.64	0.28	0.7–4.02
AA	6.1	0.004	1.76–21.2

HR hazard ratio, *CI* confidence interval

Our results clearly show that there was a robust statistical association between the *MC4R* rs489693 AA genotype and a shorter progression-free survival of patients treated with the combination of RT and TMZ. Moreover, this association was maintained also in the overall survival, indicating a statistical disadvantage for patients harboring this particular genotype if treated with this standard therapeutic approach for GBM. To our knowledge, no other data are currently available linking polymorphisms of *MC4R* gene and therapeutic efficacy or toxicity of any type of cancer, including GBM, thus representing a novel, although preliminary, discovery in the field that should be confirmed by wider and prospective pharmacogenetic trials.

One of the hallmarks of GBM is represented by the presence of necrotic areas within the tumor mass, and thus, these areas are commonly characterized by severe hypoxia. Indeed, the hypoxia-inducible factor-1 alpha (HIF-1α) has been reported to play a pivotal role in GBM development and progression because of its characteristic to induce the upregulation of numerous genes such as *VEGF* [34]. Thus, tumor cells survive acquiring molecular (e.g., VEGF secretion) or genetic changes as the result of the inadequate supply of O_2 and nutrients [35, 36]. Indeed, one of the main factors responsible for GBM treatment failure is this hypoxia, while causing apoptotic and necrotic death of normal cells permits to neoplastic cells to develop defense mechanisms [37]. Moreover, several studies have demonstrated that glioma and glioblastoma cells exhibit important resistance to apoptosis after exposure to ionizing radiation [38], as well as the key role of the expression status of B cell lymphoma-2 (Bcl-2) family proteins that are fundamental for the activation of the apoptosis pathway in GBM after TMZ administration, even in cancer stem cells [39, 40]. Besides, the antiapoptotic mechanisms of resistance [41] and also the activation of specific pathways such as Wnt-3A/β catenin, Shh, and Notch have been implicated in tumor radio- and chemoresistance [42, 43].

MC4Rs are expressed predominantly in the brain, both on neurons and astrocytes, but they were also detected in adipose tissue and in human skin melanocytes [44]. Although the brain distribution of the MC4 receptor in mice and rats has been extensively studied [44], the mapping of brain human MC4R is still an open field. Moreover, no data are available on *MC4R* gene and protein expression in GBM or other cancer cells,

although our group has demonstrated the presence of functional MC4Rs in at least two human GBM cell lines (Bocci G., unpublished data). Although at a first glance the MC4R functions have been described in completely different areas such the control of weight, it is not surprising that MC4R may share more than a simple statistical connection with the success of RT-CT therapy and/or prolonged survival in GBM. In fact, it has been demonstrated that MC4R signaling can stimulate both the survival of astrocytes, by decreasing caspase-3 activity and the expression of Bcl-2-associated X protein (Bax) and by increasing the expression of Bcl-2 [45], and the proliferation of neural stem/progenitor cell under hypoxic conditions, by triggering the canonical Wnt-3A/β-catenin and Shh signaling pathway [13]. Moreover, in an animal model of cerebral ischemia and Alzheimer's disease, the chronic treatment with the melanocortin analog [Nle(4), D-Phe(7)]α-melanocyte-stimulating hormone (NDP-α-MSH), an MC4R agonist, significantly reduced neuron death [6, 11, 46, 47]. This neuroprotection involved Bcl-2 upregulation and the decreased expression of pro-apoptotic Bax and caspase-3 activation [6, 46], as also described in a model of traumatic brain injury [48]. Moreover, proliferative effects of NDP-α-MSH have been recently reported in 7-day-old cultured astrocytes [7]. Therefore, it might be plausible that the *MC4R* rs489693 AA genetic background, although phenotypically still unknown, may be responsible, in part, for pro-survival and antiapoptotic signals to cancer cells in hypoxic conditions or after radio-chemotherapy-induced damages, determining the shorter PFS and OS in these GBM cancer patients. Conversely, in patients with a more favorable genetic profile, the MC4R survival signal to cancer cells due to the different genotypes may be reduced and, in the presence of RT and TMZ, tumor cells are less capable to proliferate or survive.

This study recruited, consecutively, 55 patients treated with RT and TMZ at our institution and other centers. An obvious limitation of this work is that the association between the PFS or OS and the *MC4R* genotype was assessed on a limited number of patients. Thus, initial data from pilot studies should be scrutinized with the most accurate statistical correction [49], as in the present study, where we applied a strict Bonferroni's correction to our data in order to avoid the risk of false-positive associations. Moreover, there are also some limitations with our findings in regard to the role of *MC4R* rs489693 as pharmacogenetic marker, which could be also a prognostic factor. Indeed, it is possible that the *MC4R* rs489693 AA genotype may be associated with a worse disease outcome, independently from the efficacy of the administration of RT and TMZ. However, the importance of pharmacogenetic pilot studies is that they may open new areas of research, such as, in this case, the possible role of MC4R in the GBM resistance to standard therapy. Furthermore, they could guide the planning of future, randomized, controlled, and multiinstitutional pharmacogenetic studies.

In conclusion, our pilot study suggests that the *MC4R* rs489693 AA genotype is significantly associated with a shorter PFS and OS in patients treated with a combined RT and TMZ schedule. These findings represent a relevant effort to identify novel clinical markers for RT–CT therapy in GBM, which can be applied in future phase III clinical trials.

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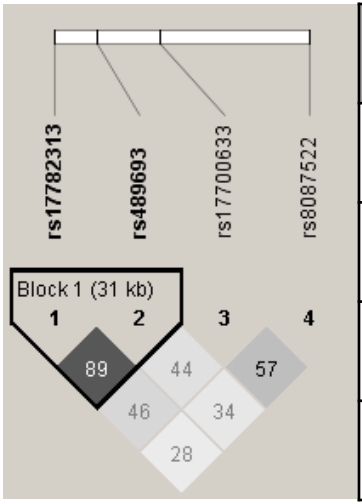
A

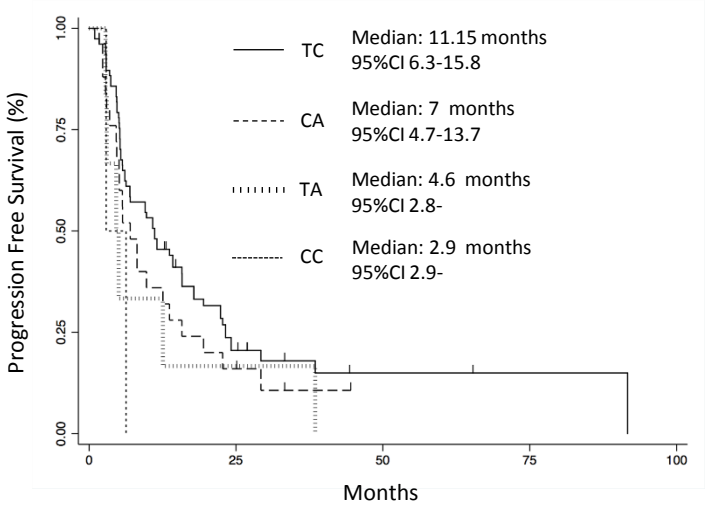


MC4R haplotypes	%
TC	69.9
CA	22.6
TA	5.6
CC	1.9

Supplementary Figure S1

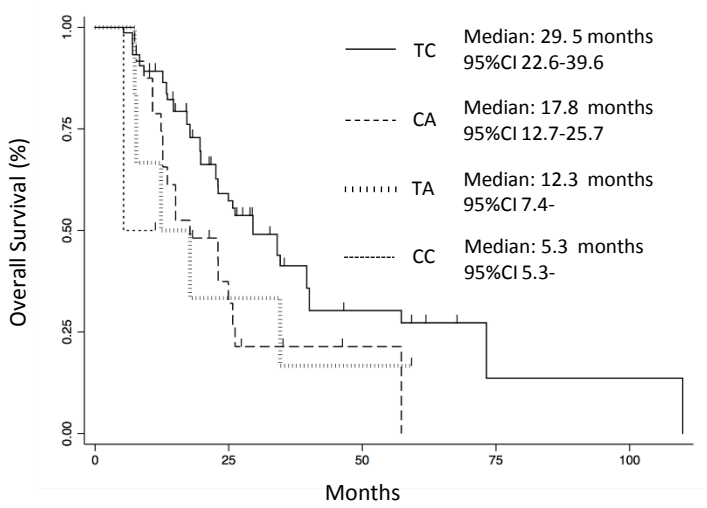
Haploview Linkage Disequilibrium Plots and Haplotype analysis in PHASE of *MC4R gene region (A). Linkage disequilibrium was measured using r2 in MC4R gene region.* Diamonds are white if *r2 = 0, varying shades of grey if 0 < r2 < 1, and are black if r2 = 1.* An haplotype analysis was performed using PHASE. Uncommon haplotypes (< 5%) were highlighted in red. None of the haplotype is significantly associated with PFS (**B**) and OS (**C**) survival in Kaplan Meier analysis for MC4R as well as in the univariate Cox model .

B



MC4R haplotypes	HR	95%CI	p
TC (reference)	1	-	-
CA	1.35	0.83-2.2	0.23
TA	1.8	0.78-4.2	0.17
CC	3.3	0.78-13.6	0.1

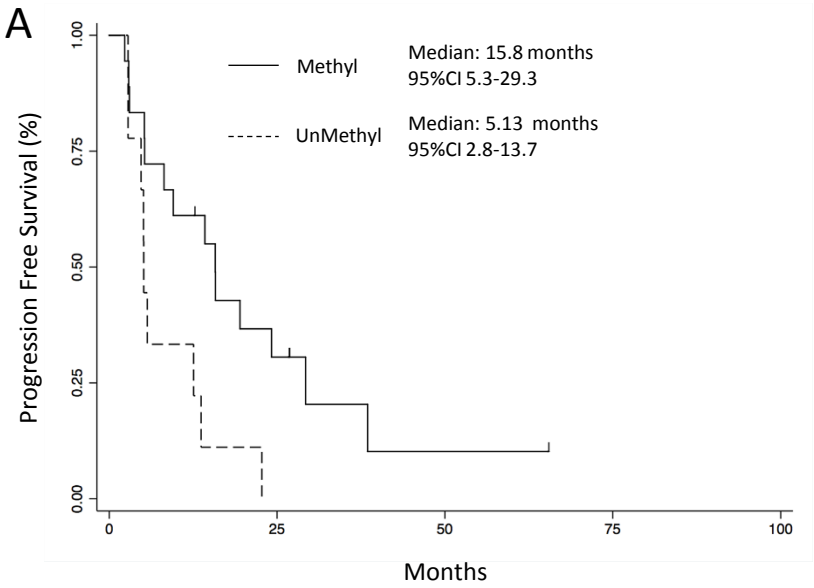
C

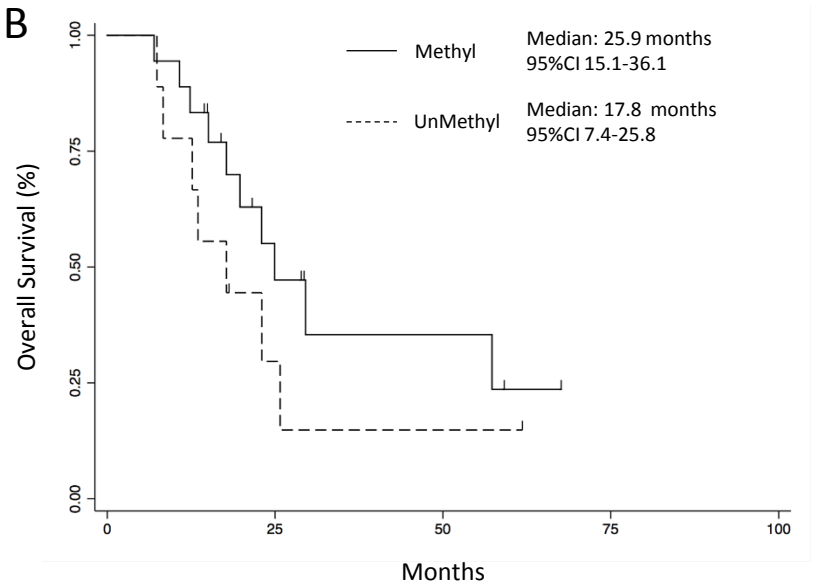


MC4R haplotypes	HR	95%CI	p
TC (reference)	1	-	-
CA	1.89	1.08-3.3	0.03
TA	1.87	0.74-4.7	0.19
CC	7.3	0.95-56.3	0.06

Supplementary Figure S2

PFS (**A**) and OS (**B**) survival in Kaplan Meier analysis for MGMT methylation as well as in the univariate Cox model .





***MC4R* haplotypes**	**HR**	**95%CI**	**p**
UnMethyl (reference)	1	-	-
Methyl	0.34	0.14-0.83	0.02

***MC4R* haplotypes**	**HR**	**95%CI**	**p**
UnMethyl (reference)	1	-	-
Methyl	0.56	0.22-1.5	0.25

Supplementary table 1 - Polymorphisms, genotypes, allele frequencies and Hardy-Weinberg Equilibrium (HWE)

ID	*Gene*	*Genotype*			*Allele*			*HWE p-value*
		G	**n**	**%**	***A***	**n**	**%**	
rs17782313	*MC4R*	TT	30	54.55	T	83	0.75	0.48
		TC	23	41.82	C	27	0.25	
		CC	2	3.64				
rs489693	*MC4R*	CC	30	54.55	C	79	0.72	0.32
		CA	19	34.55	A	31	0.28	
		AA	6	10.91				
rs8087522	*MC4R*	GG	32	58.18	G	84	0.76	1
		GA	20	36.36	A	26	0.24	
		AA	3	5.45				
rs17700633	*MC4R*	GG	35	63.64	G	89	0.81	0.66
		GA	19	34.55	A	21	0.19	
		AA	1	1.82				

Supplementary table 2. Hematological toxicities

leucopenia grade, n (%)	
0	8 (14.55)
1	17 (30.91)
2	10 (18.18)
3	2 (3.64)
4	1 (1.82)
Unknown	17 (30.91)
leucopenia, n (%)	
0-1-2	35 (63.64)
3-4	3 (5.45)
Unknown	17 (30.91)
thrombocytopenia grade, n (%)	
0	1 (1.82)
1	11 (20.00)
2	10 (18.18)
3	10 (18.18)
4	6 (10.91)
Unknown	17 (30.91)
thrombocytopenia, n (%)	
0-1-2	22 (40.00)
3-4	16 (29.09)
Unknown	17 (30.91)

Supplementary table 3. Association between clinical and pathological characteristics with PFS in the whole study cohort

	HR	*p*	*95% CI*
Weight	1.0002	0.988	0.98 - 1.02
Sex	1.008	0.98	0.56 - 1.8
Age at diagnosis	1.03	0.035	1.002 - 1.05
Previous radiotherapy	0.78	0.46	0.4 - 1.5
leucopenia, grade	0.89	0.52	0.63 - 1.26
leucopenia	0.79	0.7	0.24 - 2.6
thrombocytopenia, grade	0.88	0.44	0.64 - 1.2
thrombocytopenia	1.08	0.82	0.55 - 2.15
Performance Status	0.98	0.226	0.97 - 1.006
Lines of chemotherapy	0.72	0.057	0.51 - 1.01
Lines of chemotherapy, n			
1 (ref)	1	-	-
2	0.89	0.75	0.46 - 1.76
3	0.46	0.088	0.19 - 1.13
4	0.4	0.23	0.09 - 1.75
Frontal lobe site	0.46	0.028	0.23 - 0.92

HR, hazard ratio; CI, confidence interval. Leucopenia and thrombocypenia represents the difference between patients with ≤2 (reference) and >2, or where specified 'grade' is represented as ordinal value; Lines of chemotherapy is represented as an ordinal variable or using Line 1 as reference

Supplementary table 4. Association between clinical and pathological characteristics with OS in the whole study cohort

	HR	*p*	*95% CI*
Weight	0.99	0.75	0.97 - 1.02
Sex	0.82	0.57	0.4 - 1.64
Age at diagnosis	1.03	0.06	0.99 - 1.06
Previous radiotherapy	0.78	0.53	0.36 - 1.7
leucopenia, grade	0.93	0.74	0.61 - 1.4
leucopenia	1.38	0.6	0.41 - 4.7
thrombocytopenia, grade	1.06	0.75	0.73 - 1.54
thrombocytopenia	1.12	0.79	0.5 - 2.5
Performance Status	0.98	0.03	0.96 - 0.99
Lines of chemotherapy	0.6	0.031	0.38 - 0.95
Lines of chemotherapy, n			
1 (ref)	1	-	-
2	0.76	0.48	0.34 - 1.65
3	0.26	0.036	0.07 - 0.92
4	0.37	0.333	0.05 - 2.8
Frontal lobe site	0.38	0.026	0.16 - 0.89

HR, hazard ratio; CI, confidence interval. Leucopenia and thrombocytopenia represents the difference between patients with ≤2 (reference) and >2, or where specified 'grade' is represented as ordinal value. Lines of chemotherapy is represented as an ordinal variable or using Line 1 as reference.

Supplementary table 5. Model selection using Bayesian Information Criterion (BIC)

Age at diagnosis	Performance Status	Lines of chemotherapy	Frontal lobe site	rs489693 additive	rs489693 dominant	BIC	ΔBIC
	x		x		x	166.04	Δ
	x		x	x		168.60	2.56
x	x		x			169.29	3.25
	x	x	x			170.54	4.50
x	x				x	176.55	10.51
	x	x			x	178.58	12.54
x	x	x				179.39	13.35
x	x			x		180.21	14.17
		x	x		x	180.78	14.74
	x	x		x		182.34	16.30
		x	x	x		184.09	18.05
x		x	x			190.19	24.15
x			x		x	191.44	25.40
x		x			x	192.12	26.08
x			x	x		194.24	28.20
x		x		x		196.05	30.01

Lines of chemotherapy is represented as an ordinal variable or using Line 1 as reference.

Each line represent a different combination. The Bayesian Information Criterion (BIC) was calculated for each model and the one with the lowest BIC is preferred. The strength of the evidence against the model with the higher BIC value can be summarized as follows: ΔBIC - 0 to 2, not worth more than a bare mention; ΔBIC - 2 to 6, positive evidence; ΔBIC - 6 to 10, strong evidence; ΔBIC - >10, very strong evidence