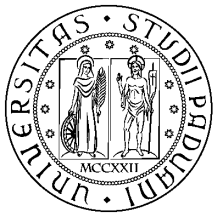

UNIVERSITÀ
DEGLI STUDI
DI PADOVA

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Scuola di Dottorato di Ricerca in Oncologia e Oncologia Chirurgica

Indirizzo Comune

Ciclo XXVI

2-Hydroxyglutarate as a biomarker in glioma patients

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Abstract

Background: mutation of IDH1 gene is a prognostic factor and a diagnostic hallmark of gliomas. Mutant IDH1 enzyme can convert α-KG into 2-Hydroxyglutarate (2HG) and mutated gliomas have elevated amounts of intracellular 2HG. Since 2HG is a small molecule it seems possible that it could reach the systemic circulation and to be excreted by urine. And so, we analyzed 2HG concentration in plasma and urine in glioma patients to identify a surrogate biomarker of IDH1 gene mutation.

Materials and Methods: All patients had a prior histological confirmation of glioma, a recent brain MRI (within 2 weeks) showing the neoplastic lesions. The exclusion criteria were any chemotherapy performed within 28 days prior, other neoplastic and metabolic diseases. Plasma and urine samples were taken from all patients and 2HG concentrations determined by liquid chromatography tandem mass spectrometry; exon 4 of IDH1 genes were analyzed by Sanger sequencing; differences in metabolite concentrations between mutant and wild-type IDH1 patients were examined with the Mann-Whitney U test for non-parametric data; Student's t-test was used to compare parametric data. ROC curve was used to evaluate the cut off value of the 2HG biomarker.

Results: 84 patients were enrolled: 38 with IDH1 mutated and 46 IDH1 wild-type. All the mutations were R132H. Among patients with mutant IDH1 we had 21 high-grade gliomas (HGG) and 17 low-grade gliomas (LGG); among patients with IDH wild-type we had 35 HGG and 11 LGG.. In all patients we analyzed the mean 2HG concentration in plasma (P_2HG), in urine (U_2HG) and the ratio between P_2HG and U_2HG (R_2HG).

We found an important significant difference in R_2HG between glioma patients with and without IDH1 mutation (22.2 versus 15.6, respectively, $p<0.0001$). The optimal cut-off value of R_2HG to identify glioma patients with and without IDH mutation was 19 (sensitivity 63%, specificity 76%, accuracy 70%); in only PTS with HGG the optimal cut-off value was 20 (sensitivity 76%, specificity 89%, accuracy 84%, positive predictive value 80%, negative predictive value 86%). No associations between the grade or size of tumor and R_2HG were found. In 7 patients with high-grade gliomas we found a correlation between R_2HG value and response to treatment.

Conclusions: analyzing R_2HG derived from individual plasma and urine 2HG levels is possible discriminate glioma patients with and without IDH mutation, in particular in high grade gliomas. Moreover, a larger samples need to be analyzed to investigate this method in patients follow-up for recurrence detection and to monitor treatment efficacy.

Abstract

Background: la mutazione del gene IDH1 rappresenta un importante fattore prognostico e diagnostico per i tumori gliali. L'enzima IDH1 avente la mutazione ha la capacità di convertire α-KG in 2-Idrossiglutarato (2HG) e i gliomi mutati hanno una elevata concentrazione di 2HG all'interno delle cellule tumorali. Poichè 2HG è una piccola molecola, tale metabolita potrebbe raggiungere la circolazione sistemica ed essere escreta con le urine. Per tale ragione, nel nostro studio abbiamo analizzato la concentrazione di 2HG nel plasma e nelle urine nei pazienti con glioma per identificare un biomarcatore surrogato della presenza della mutazione IDH1.

Materiali e Metodi: per l'arruolamento, tutti i pazienti dovevano avere avuto una precedente conferma istologica di glioma, una recente risonanza magnetica cerebrale (entro 2 settimane) mostrante la lesione tumorale. Qualsiasi chemioterapia eseguita nei 28 giorni precedenti l'analisi del metabolita, la presenza di altre malattie tumorali e malattie metaboliche escludevano l'arruolamnto del paziente. Campioni plasmatici e urinari sono stati ottenuti da tutti i pazienti e le concentrazioni di 2HG ottenute mediante cromatografia liquida-spettrometria di massa; il test di Mann-Whitney è stato usato per calcolare le differenze di concentrazione dei metaboliti tra pazienti con IDH1 mutato e non-mutato, per dati non parametrici; il test di Student per comparare dati parametrici. La curva ROC è stata usata per calcolare il valore di cut-off del 2HG come biomarcatore.

Risultati: sono stati arruolati 84 pazienti: 38 con IDH1 mutato e 46 con IDH1 wild-type. Tutte le mutazioni sono state R132H. Tra i pazienti con mutazione IDH1 abbiamo avuto 21 gliomi ad alto grado (HGG) e 17 gliomi a basso grado (LGG). Tra i pazienti con IDH1 wild-type abbiamo avuto 35 pazienti con HGG e 11 con LGG. In

tutti i pazienti abbiamo analizzato la concentrazione media di 2HG nel plasma (P_2HG), nell'urina (U_2HG) e il rapporto tra la concentrazione plasmatica e urinaria (R_2HG). E' emersa una importante differenza statisticamente significativa per l'R_2HG tra pazienti con e senza mutazione dell'IDH1 (22.2 verso 15.6, $p<0.0001$). Il cut-off ottimale di R_2HG per identificare lo stato mutazionale di IDH1 nei pazienti con glioma è risultato essere 19 (sensibilità 63%, specificità 76%, accuratezza 70%); nei soli pazienti con glioma ad alto grado il cut-off ottimale è risultato essere 20 (sensibilità 76%, specificità 89%, accuratezza 84%, valore predittivo positivo 80%, valore predittivo negativo 86%). Non è emersa nessuna associazione tra il grado o la dimensione del tumore con il valore di R_2HG. In 7 pazienti con glioma ad alto grado analizzati abbiamo, inoltre, trovato una correlazione tra il valore di R_2HG e la risposta al trattamento.

Conclusioni: attraverso l'analisi di R_2HG, derivato dalla concentrazione plasmatica e urinaria di 2HG, è possibile discriminare gliomi con e senza mutazione IDH1, soprattutto in gliomi di alto grado. Occorrerà analizzare un campione più grande di pazienti con glioma per investigare tale metodica anche nel follow up allo scopo di individuare precocemente la recidiva di malattia e per monitorare l'efficacia del trattamento.

INDICE

INTRODUCTION

Epidemiology and Classification of brain tumors

Primary malignant brain tumors account for 1.4% of all cancers and are the cause of 2.4% of all cancer deaths in Western countries(1). The overall average annual age-adjusted incidence rate for 2005-2009 for primary brain and central nervous system (CNS) tumors was 20.5 per 100,000. The overall incidence rate was 5.1 per 100,000 for children and 26.8 per 100,000 for adults. In addition, the average annual age-adjusted incidence rates of all primary malignant brain and CNS tumors ranged from 4.9 to 8.9 per 100,000 and the averaged annual age-adjusted incidence rates of all primary non-malignant brain and CNS tumors ranged from 8.9 to 19.02 per 100,000. Among adults, the incidence rates ranged from 5.8 to 11.7 per 100,000 for malignant tumors and from 11.9 to 25.94 per 100,000 for non-malignant tumors.

For those with malignant behaviour, the incidence rate was highest for glioblastoma (3.19 per 100,000) followed by diffuse astrocytoma (0.58 per 100,000), and lymphoma (0.45 per 100,000). Meningioma (7.1 per 100,000), tumors of the pituitary (2.93 per 100,000), and nerve sheath (1.69 per 100,000) tumors were the non-malignant histologies with the highest incidence rates(1). Glioblastomas account for 16% of all primary brain tumors and are more common in older adults. The incidence of glioblastoma increases with increasing age, with rates highest in 75 to 84 years olds. Moreover, glioblastomas are 1.6 times more common in males and are two to three times higher among whites as compared to black race groups.

The estimated five-and ten year relative survival rates for malignant brain and CNS tumors are 33.8% and 28%, respectively. However, there is a large variation in

survival estimates depending upon tumor histologies. For example, five-year survival rates are 94% for pilocytic astrocytomas but are less than 5% for glioblastomas. Survival generally decreases with older age at diagnosis.

However, the most common primary malignant brain tumors are of glial origin and arise from astrocytes (astrocytomas), oligodendrocytes (oligodendrogliomas), or ependymal cells (ependymomas). The World Health Organization (WHO) system is the most frequently used classification schema and is based on standard histologic criteria(2). According to WHO criteria, up to 80% of gliomas are either WHO grade III (anaplastic astrocytoma or oligodendrogliomas) or WHO grade IV (glioblastomas). Pilocytic astrocytoma, which occur most commonly in children, are considered WHO grade I, and low-grade astrocytomas and oligodendrogliomas are considered WHO grade II. Recent studies have reported the median overall survival for patients with glioblastoma to be close to 20 months, in contrast to the much longer median survival of just over 7 years for patients with low-grade astrocytomas(3).

In addition to histologic classification of gliomas, interest in the molecular characterization of these tumors is increasing. For example, glioblastomas can be divided into primary or secondary GBM. Primary GBM tends to occur in older patients and is characterized by EGFR amplification, PTEN mutation, and p16 deletion. Secondary GBM arises from lower grade gliomas and occurs in younger patients who have mutation in TP53 and IDH1, PDGFRB overexpression, and aberrant signalling of the Rb pathway.

Diagnosis

The signs and symptoms caused by brain tumors are related to their location and rate of growth and are therefore quite variable. A rapidly growing relatively circumscribed tumor may result in acute focal deficits, whereas a slow-growing diffuse mass can cause gradual changes in behaviour or cognition. Intractable headache, new-onset seizures, cognitive dysfunction, nausea, and vomiting are frequent accompaniments of brain tumors but are not specific, so clinical context remains important. Once a tumor is suspected, neuroimaging with contrast-enhanced magnetic resonance imaging (MRI) will confirm the presence of a mass lesion. Imaging features such as enhancement pattern and location and even additional imaging data such as MRI spectroscopy can suggest but not definitively provide a diagnosis, which can be obtained only by examination of tissue.

Treatments for gliomas

The mainstays of tumor-directed treatment for gliomas include resection, radiation, and chemotherapy either alone or in combination, and the choice is based on histology, tumor grade, and general medical condition. For some gliomas Level I evidence supports a specific therapeutic modality, but treatment decision are often based on results from single-arm phase II studies that compare results with historical controls. However, recent advances in understanding the molecular genetics of gliomas and in the elucidation of tumor-specific prognostic and predictive markers are leading the way for development of patient-specific treatment strategies.

- *Surgery*

  The initial therapeutic approach for gliomas is surgery. Surgery not only confirms the diagnosis and provides tissue for genetic studies but also can improve symptoms by relieving mass effect and edema. Surgery is not required in some intrinsic tumors of the brainstem that are not amenable to a surgical procedure. Cumulative data suggest that the extent of resection impacts outcome for all grade of gliomas: in fact, longer survivals are seen in those patients who undergo near total or gross total resection(4). In these studies, the extent of resection was usually defined by the presence or absence of residual contrast enhancement on MRI taken within 48 hours of resection.

- *Radiation*

  Radiation is the most common post-surgical treatment modality for all grades of gliomas and is at the times given with concurrent chemotherapy. Radiation is delivered to a limited field that targets the area of the lesion and usually a 1 to 3 cm margin. The total dose delivered increases with glioma grade. For glioblastoma, daily fractions of 1.8 to 2 Gy are delivered over 6 weeks for a total dose close to and rarely exceeding 60 Gy. Studies have not shown a benefit of higher doses or of alternative fraction schedules.

- *Chemotherapy*

  Chemotherapy is playing an increasing role in the treatment of gliomas. While a large variety of agents have been evaluated, currently the most commonly used chemotherapeutic is temozolomide, an oral cytotoxic DNA-alkylating agent. The benefit of temozolomide for glioblastoma in particular was shown in a phase III trial in which patients with newly diagnosed glioblastoma were

randomized after surgery to receive either treatment with external beam radiation therapy alone or radiation therapy with concurrent temozolomide(3). This study demonstrated that chemoradiation with adjuvant temozolomide resulted in a significant improvement in median overall survival compared with radiation only (14.6 months versus 12.1 months) and a significant increase in 2-year survival (26.5% versus 10.4%). A recent evaluation of these patients demonstrated that the benefit of this regimen lasted throughout 5 years of follow up(5). A companion study demonstrated that in the subgroup of patients whose tumors were shown to have low levels of O6-methylguanine DNA methyltransferase (MGMT), the enzyme that repairs DNA damage due to temozolomide, overall survival was 46% at 2 years and 14% at 5 years(6).

Regarding grade III gliomas, those patients with grade III oligodendrogliomas have increased response to therapy and length of survival compared with patients with grade III astrocytomas. These improved outcomes are strongly associated with the loss of heterozygosity of chromosome 1p and 19q, which is found in about 80% of cases (7). The deletion is due to an unbalanced translocation of 19q to 1p. A recent study demonstrated that patients with grade III oligodendroglioma and deletion 1p19q have a longer progression-free survival and overall survival when treated with radiation therapy and chemotherapy with procarbazine, lomustine and vincristine than patients without the deletion(8).

In the last years, new targeted drugs have been investigated for high grade gliomas, such as antiangiogenic drugs (bevacizumab), anti-integrin drug (cilengitide), mTOR inhibitors (sorafenib, everolimus) but no drug showed an

interesting efficacy but bevacizumab that demonstrated interesting results in some phase II studies in recurrent glioblastoma patients(9). To date, a trial is ongoing to evaluate the efficacy of bevacizumab as first line treatment with radiation therapy and temozolomide in glioblastoma.

Regarding low grade gliomas, the usefulness of chemotherapy for patients progressing after surgery and radiotherapy is well established. PCV (Procarbazine, lomustine, vincristine) and temozolomide yield similar objective response rates and duration of response (10-24 months), with a toxicity profile favouring temozolomide(10). Chemotherapy as initial treatment after surgery has been investigated either in association with radiotherapy or alone, delaying radiation as consolidation therapy or at tumor progression. The studies have included "high-risk patients", defined by the presence of one or more poor prognostic factors, such as age over 40 years, large initial tumor volume, presence of residual tumor after resection, astrocytic histology, neurological symptoms, and progression on MRI. Regarding anaplastic ependymomas, temozolomide alone or in combination to cisplatin, can be efficacy(11).

Predictive and prognostic factors in gliomas

The WHO classification has various limitations due to the fact that classification is still based on subjective criteria, both for phenotype determination and grading, and the fact that one histological subtype encompasses different molecular subtype with different prognoses. Indeed, it is becoming increasingly clear that tumors that share identical histopathologic features can actually represent multiple distinct molecular phenotypes. A more

detailed molecular understanding of these tumors is thus crucial to improve classification, to better predict outcome, to better stratify patients included in clinical trials and, finally, to tailor specific treatment to individual tumor types or patients. To date, three biomarkers have been identified as potent prognostic factors in gliomas: codeletion of chromosome arms 1p and 19q, O6-methylguanine-DNA methyltransferase (MGMT), isocitrate dehydrogenase (IDH) (12) .

- *Codeletion of chromosome arms 1p and 19q*

  The combined loss of one copy of chromosome arms 1p and 19q occurs in the majiority of oligodendrogliomas and anaplastic oligodendrogliomas, as well as in important number of oligoastrocytomas and anaplastic oligoastrocytomas. This signature is a strong prognostic factors and also a predictive factor of response to chemotherapy as well as radiotherapy (7). In both low-grade and anaplastic oligodendrogliomas, 1p/19q codeletion is predictive of longer progression-free survival and overall survival after chemotherapy, radiotherapy or both. The median survival is 12-15 years in low-grade oligodendrogliomas and more than 7 years in anaplastic oligodendrogliomas with 1p/19q codeletion, versus 5-8 years and 2-3 years, respectively, in the absence of grade I (12). The 1p/19q codeletion is mutually exclusive with TP53 mutation and EGFR amplification, frequently associated with MGMT promoter methylation, and always associated with IDH1 or IDH2 mutation. MGMT promoter hypermethylation is significantly more frequent and the percentage of methylated CpG site was significantly higher in 1p/19q codeletion tumors compared with 1p and/or 19q intact. The high frequency of MGMT

promoter methylation in 1p/19q codeletion gliomas might partly explain their chemosensitivity. Because it is a strong prognostic factor in both low-grade and anaplastic oligodendrogliomas, and is predictive of response to both chemo-and radiotherapy, 1p/19q codeletion is currently used as a selection criteria.

- *O6-methylguanine-DNA methyltranseferase (MGMT)*

O6-methylguanine-DNA methyltransferase is a DNA-repair enzyme that removes alkyl groups from O6 position of guanine, one of the most frequent sites of DNA alkylation induced by chemotherapeutic agents. By suppressing these alkyl groups, MGMT is hypothesized to preclude the formation of crosslinks between adjacent strands of DNA, thereby limiting the effectiveness of alkylating agents. Based on initial studies in glioblastomas and on its biological role, MGMT promoter methylation has been initially mostly considered as a predictive factor of response to chemotherapy, presumably because MGMT repairs temozolomide-induced DNA damage, although this has not been formally demonstrated. In fact, recent studies in anaplastic gliomas and glioblastomas showed that MGMT promoter methylation is also predictive of better response to radiotherapy, suggesting that it may be a marker of better therapeutic response (13). The methylation status of the MGMT promoter was investigated in the EORTC clinical trial testing concomitant and adjuvant temozolomide versus radiotherapy alone in glioblastoma patients (6). Whatever the treatment, MGMT promoter methylation was an independent prognostic factor of favourable outcome, and was associated with longer progression-free survival and overall survival. The benefit of concomitant and adjuvant

temozolomide ro radiotherapy was clear for patients with MGMT promoter methylation (overall survival 27.2% at 2 years, 16% at 3 years and 9.8% at 5 years, versus 10.9, 4.4 and 3% for patients receiving radiotherapy alone, respectively) but not for patients without MGMT promoter methylation (5, 6).

The prognostic impact of MGMT methylation status has also investigated in gliomas of lower grades. Surprisingly, MGMT promoter methylation was reported to be prognostic but not predictive in anaplastic oligodendroglial tumor patients treated with radiotherapy and adjuvant PCV versus radiotherapy alone (14). Similarly, the NOA-04 trial reported an important difference in progression-free survival between patients with or without MGMT promoter methylation, even when treated with radiotherapy alone (15).However, because it is an important prognostic and predictive factor in gliomas, ongoing trials use MGMT promoter methylation status to stratify patients.

Isocitrate dehydrogenase gene mutations (IDH)

Mutation of the isocitrate dehydrogenase 1 gene in gliomas is undoubtedly one of the most ground-breaking discoveries in the field of neuro-oncology in recent years. These mutations were first discovered in a subset of glioblastomas in a study in which 22 glioblastoma specimens (16). Further analysis of IDH1 in an extended tumor series confirmed that IDH1 mutations occurs in 12% of all glioblastomas. The types of tumor in which IDH 1/2 mutations prevalently occur are very specific. Among tumors of the central nervous system, mutations of IDH are found in approximately 50-90%

of adult astrocytomas and oligodendrogliomas of WHO grades II and III, and secondary glioblastomas. In contrast, IDH mutations occur only in a small subset (3-16%) of primary glioblastomas. (17). All reported IDH mutations are missense. Almost all IDH1 mutations in gliomas occur at codon 132 encoding arginine (R132), with a very rare exception where arginine 100 is replaced with glutamine (R100Q) in a few tumors (18). Approximately 90% of all IDH1 mutations are G to A transition at the second nucleotide of codon 132 resulting in the substitution of arginine by histidine (R132H). Other rare types of IDH1 mutations include substitution of arginine with cysteine, serine, glycine, or leucine. Mutations of IDH2 in gliomas are much less common than those of IDH1. Mutations of IDH1 and IDH2 are mutually exclusive in gliomas, suggesting that the consequences of the mutations in these two genes might be equivalent. IDH mutations almost always occur hemizygously.

Another feature observed in gliomas is that almost all tumors with IDH mutations also harbour wither TP53 mutations or total 1p/19q loss.n The concurrent IDH and TP53 mutations are typically observed in astrocytic tumors of grades II and III and in secondary glioblastomas. The combination of IDH mutations and total 1p/19q loss, is predominantly found among oligodendrogliomas of grades II and III. Oligoastrocytic tumors have either combined TP53 and IDH mutations, or total 1p/19q loss and IDH mutations. Because the presence of the TP53 mutation or total 1p/19q loss has long been regarded as an indicator of either astrocytic or oligodendroglial tumors and, therefore, uniquely contributes to the distinct molecular pathogenesis of these tumors, the fact that these two types of tumors always share the same genetic change. This has led to the idea that astrocytic and oligodendroglial tumors may share common progenitor cells. These cells would first acquire an IDH mutation and subsequently

progress either to astrocytomas by obtaining TP53 mutations or to oligodendrogliomas by developing total 1p/19q loss (19) (see Figure 1).

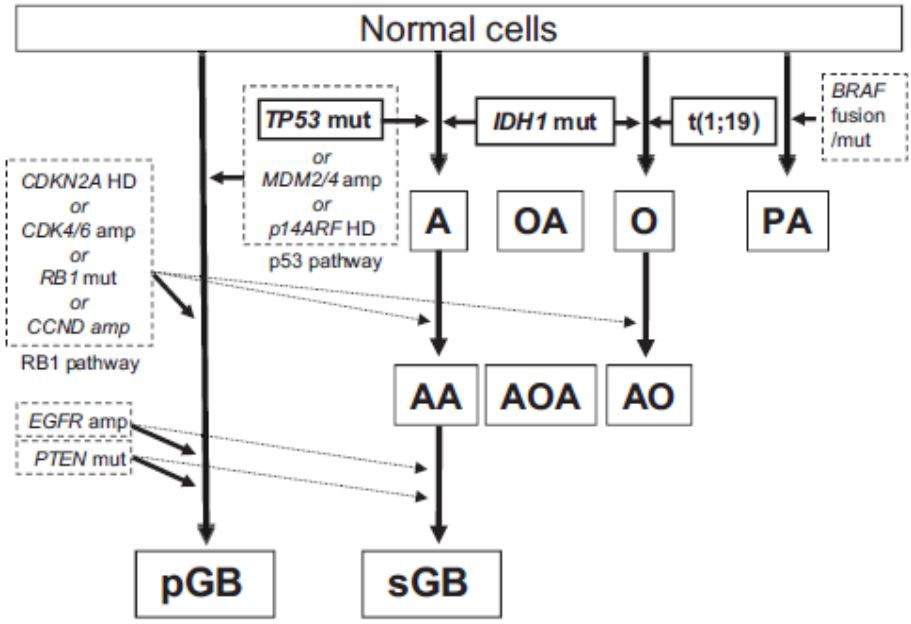

Figure 1. Model of development of glial tumors (19).

Supporting evidence for this theory comes from a study in which a series of paired initial and recurrent tumors was screened for the presence of IDH1 mutations, TP53 mutations, and 1p19q deletions. Four diffuse astrocytomas which initially had IDH1 mutations alone were found to have developed TP53 mutations at recurrence. Likewise, three oligodendrogliomas had 1p19q loss upon recurrence, while retaining the same IDH1 mutation that was present initially in the original tumors. No tumor has ever been shown to develop IDH1 mutation after acquisition of a TP53 mutation or after 1p19q loss. These findings validate the chronological order proposed for the genetic events observed, placing IDH1 mutations as the earliest genetic changes common to both astrocytic and oligodendroglial tumors (20).

There are three isozymes for IDH: IDH1, IDH2 and IDH3. The main function common to all isozymes is to catalyze the oxidative decarboxylation of isocitrate into α-ketoglutarate (aKG) (17). IDH1 is located in the cytoplasm and peroxisomes where it participates in lipid metabolism and glucose sensing. IDH2 and IDH3 are located in mitochondria and involved in the Krebs cycle. Mutated IDH1 or IDH2 have significantly reduced enzymatic activity in catalysis of conversion of isocitrate to aKG, irrespective of the nature of the substituted amino acid (19). The most prominent consequence universally acknowledged in all types of IDH mutation is that the altered enzyme acquires neomorphic activity to reduce aKG into D-2-hydroxyglutarate (D-2HG) in an NADPH-dependent manner. The mutant IDH produces only D-2HG but not its enantiomer L-2-hydroxyglutarate (21). It was demonstrated that 2HG levels are elevated in human gliomas samples; in particular, gliomas with wild-type IDH1 had over 100-fold less 2HG (21). Regardless of the mechanism, it seems likely that the gain-of-function ability of cells to produce 2HG as a result of R132 mutations in IDH1 contributes to tumorigenesis. In turns, hypoxia-inducible factor (HIF-1) levels increased, which was attributed to release from negative regulation by aKG, which is required for prolylhydroxylase activity. Prolylhydroxylases promote the degradationof HIF-1. Increased HIF-1 activity has been shown to be of great importance in the biology of glioblastoma; moreover, IDH enzymes with altered activity profiles may directly or indirectly affect other metabolic pathways, including NADP-dependent pathways such as the pentose phosphate pathway, intracellular base acid balances, and antioxidant properties (22).

However, high levels of 2HG have been demonstrated in tumor tissue of glioma patients with IDH1 mutation. Since 2HG is a small molecule it seems possible that it could reach the systemic circulation and to be excreted by urine and that alterated

2HG serum or urine levels may help to identify patients harboring IDH1 mutated gliomas. In the study, we analyzed 2HG concentration in plasma and urine in glioma patients to identify a biomarker of IDH gene mutation; moreover, we investigated the correlation between tumor volume and grade with 2HG concentration in plasma and urine. The identification of a reliable biomarker of IDH gene mutation in bodily fluids (urine and plasma) would be useful in patients who are not amenable to biopsy

MATERIALS AND METHODS

Patients enrolled in this study development at the Medical Oncology 1 Unit, Venetian Oncology Institute – IRCCS, Padua, Italy, must meet the following inclusion and exclusion criteria.

Among inclusion criteria:

- A prior biopsy of the brain tumor and histological confirmation of glioma
- Neoplastic tissue available for analysis of IDH1/2 genes by PCR and sequence analysis
- A recent brain MRI (within 2 weeks) showing the neoplastic lesion
- Written consent

Among excluxion criteria:

- Absence of neoplastic lesions on brain MRI
- Any chemotherapy performed within 28 days prior
- Other neoplastic and metabolic diseases
- Renal and/or liver failure

Plasma and overnight-urine samples were taken from all the patients. To analyze IDH1 gene mutations, DNA isolated from formalin-fixed tumor tissues was subjected to PCR using primer pairs specific for **exon 4 of IDH1**. The amplified products were subjected to sequencing analysis by fluorescent capillary electrophoresis (ABI PRISM 310 genetic analyzer; Applied Biosystem, Carlsbad, California, USA).

Metabolite concentrations of 2-hydroxyglutarate in urine and plasma were determined by liquid chromatography tandem mass spectrometry (LC-MS/MS), at

Experimental and Clinical Pharmacology Unit, National Cancer Insitute, Aviano, Italy. Differences in metabolite concentrations between mutant and wild-type IDH1 patients were examined with the Mann-Whitney U test for non-parametric data; Student's t-test was used to compare parametric data. Spearman's rank correlation was used to analyze association between tumor size and metabolite concentration. **Tumor volume** was estimated based on Flair imaging for LGGs and on contrast-enhanced tumor area for HGGs using the formula for an ellipsoid "π (a x b x c)/6"; All p-values were two-tailed, and were considered significant when lower than 0.05.

The reciver operating characteristic (ROC) curve was performed in order to determine the cut-off value of 2HG biomarker. The optimal cut-off calue was determined at the point on ROC curve at which (sensitivity + specificity-1) was maximized (Youden Index).

All statistical analyses were carried out using SPSS statistical software, version 18.0 (SPSS Inc, Chicago, Illinois, USA).

RESULTS

From January 2011 to May 2013 we enrolled 84 patients: 38 patients with mutant IDH1 and 46 patients with wild-type IDH1 (see Table 1).

	mut IDH1	**wt IDH1**
Number	38	46
Sex	17 F, 21 M	22 F, 24 M
Average Age (years)	51	62
Hystology	21 HGG	35 HGG
	17 LGG	11 LGG
Type of Mutation	R132H	-
HGG median volume	2.8 cm^3 (0.6-47.1)	3.8 cm^3 (0.6-131.9)
LGG average volume	9.4 cm^3 (3.1-81.6)	22 cm^3 (6.3-150.7)

Table 1. Characteristics of patients with mutant and wild-type IDH1. IDH1: isocitrate dehydrogenase 1; mut: mutant; wt: wild-type; HGG:high grade gliomas; LGG: low grade gliomas.

Among patients with mutant IDH1 we had: 17 females and 21 males; the average age was 51 years; 21 patients had high-grade gliomas and 17 had low-grade gliomas; the average size of the tumor was 9,2 cm^3. The characteristics of the wild-type IDH1 patients were: 22 females and 24 males, the average age was 62 years; 35 patients with high-grade gliomas and 11 patients with low-grade gliomas; the average size of the tumor was 38.5 cm^3.

The concentration of 2HG in plasma was not significantly different between the two groups (p=0.9): the mean 2HG concentration was 97±44.11 ng/mL in patients with wild-type IDH1 and 97.2±60.4 ng/mL in the other patients; the median 2HG concentration was 80.8 ng/mL (range 45.3-235.0) and 79.1 ng/mL (range 51.2-405.0) in patients with wild-type and mutant IDH1, respectively (see Figure 2).

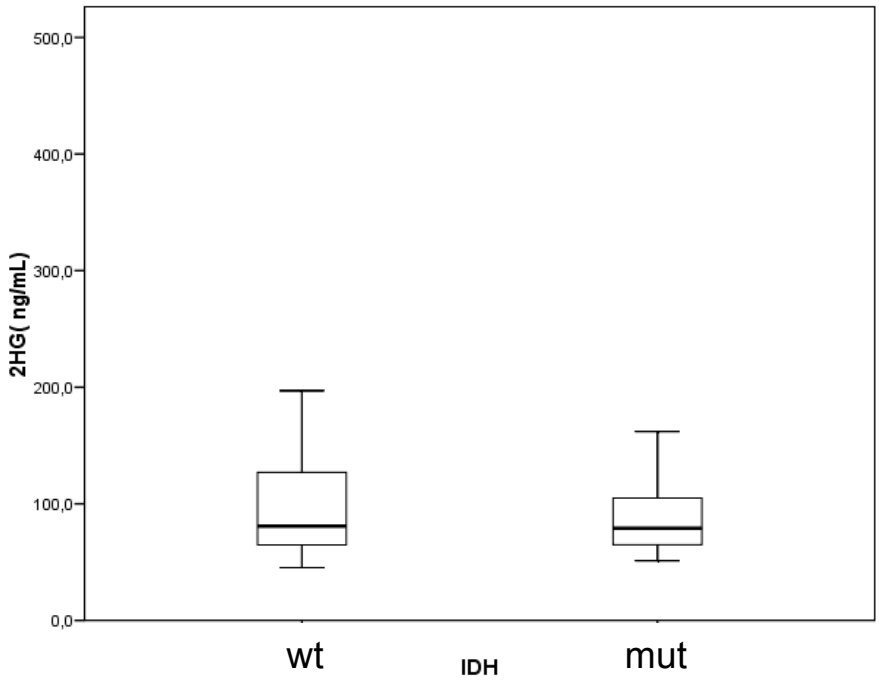

Figure 2. 2HG concentration in plasma in patients with mutant IDH and wild-type IDH.

Regarding 2HG concentration in urine (normalized by creatinine concentration), we found a statistically significant difference (p=0.002): the mean 2HG concentration was 7.3±4.5 μg/mg in patients with wild-type IDH1 and 4.6±2.0 μg/mg in the other patients; the median 2HG concentration was 5.6 μg/mg (range 1.8-22.6) and 4.1 μg/mg (range 2.2-12.3) in patients with wild-type and mutant IDH1, respectively (see Figure 3).

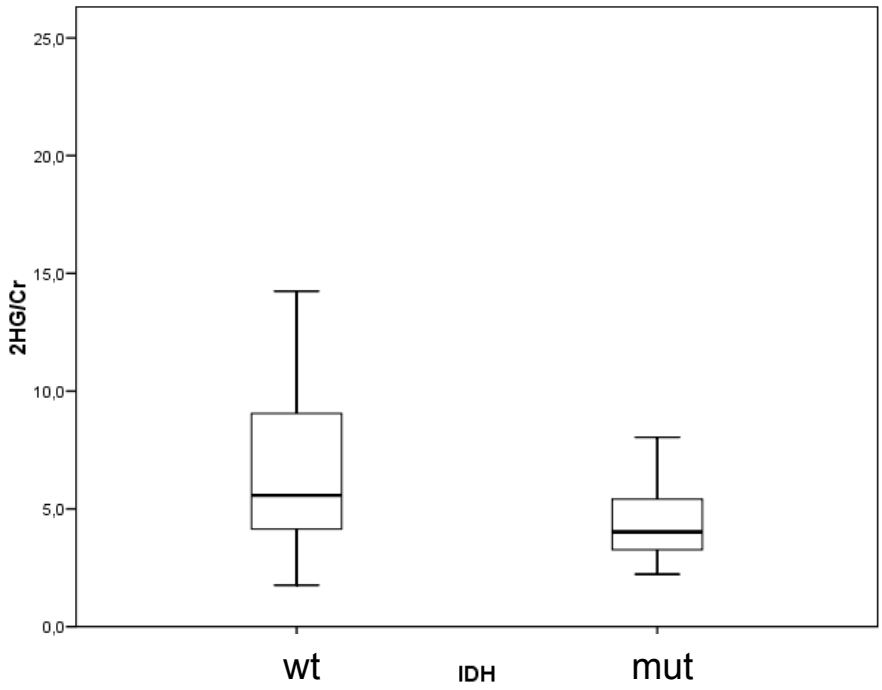

Figure 3. 2HG concentration in urine in patients with mutant IDH and wild-type IDH

Moreover, we analyzed the ratio between the concentration of 2HG in plasma and urine (R_2HG). We found an important difference between the two group ($p<0.0001$): the mean R_2HG concentration was 15.6±6.8 in patients with wild-type IDH1 and 2.2±8.7 in the other patients; the median R_2HG concentration was 15.1 (range 4.5-35.4) and 21.1 (range 7.6-42.4) in patients with wild-type and mutant IDH1, respectively (see Figure 4).

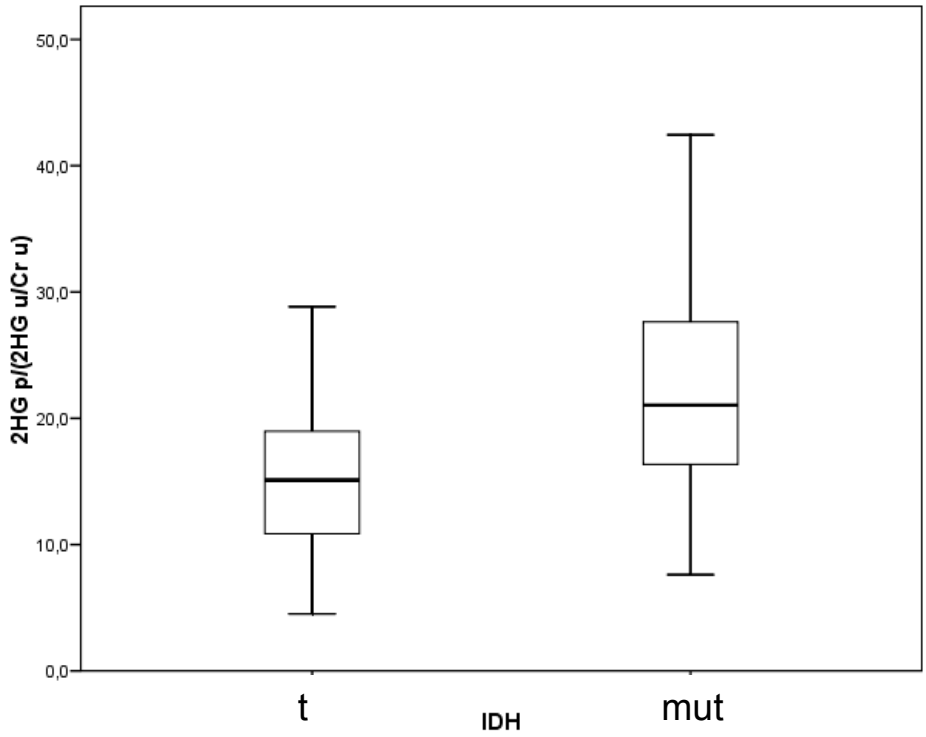

Figure 4. Ratio between R_2HG concentration in plasma and in urine in all glioma patients with mutant IDH and wild-type IDH

Analyzing the metabolite concentration in only 56 patients with high-grade gliomas we found that the R_2HG concentration had a higher difference that in all glioma patients: the mean R_2HG concentration was 15.3±6.5 in patients with wild-type IDH1 and 24.9±9.1 in the other patients; the median R_2HG concentration was 14.9 (range 4.5-35.4) and 23.1 (range 7.6-42.4) in patients with wild-type and mutant IDH1, respectively (see Figure 5).

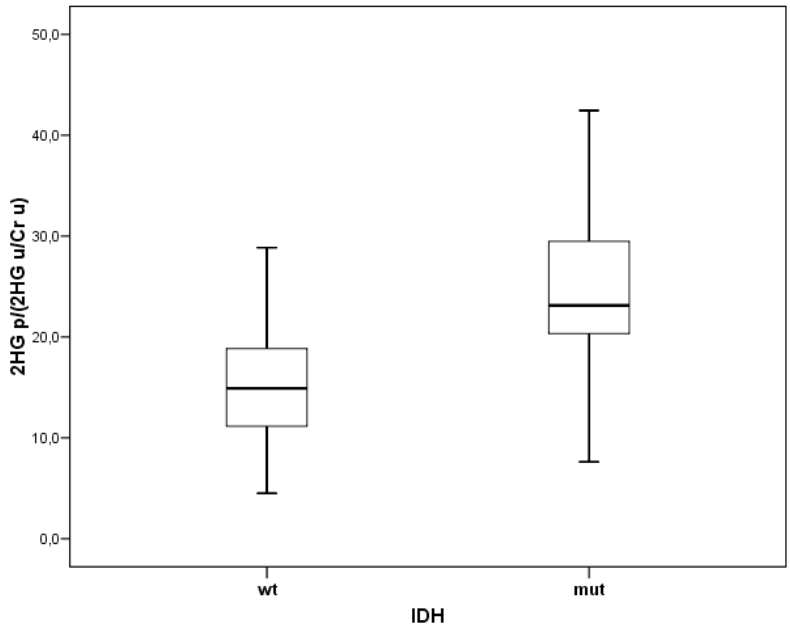

Figure 5. Ratio between R_2HG concentration in plasma and in urine in HGG patients with mutant IDH and wild-type IDH

The ROC curve analysis was performed to determine the cut off value of R_2HG. In all gliomas patients, the optimal cut-off value of R_2HG to discriminate patients with and without IDH1 mutation was 19 with which sensitivity and specificity was 63% and 76%, respectively; its accuracy was 70% (see Figure 6).

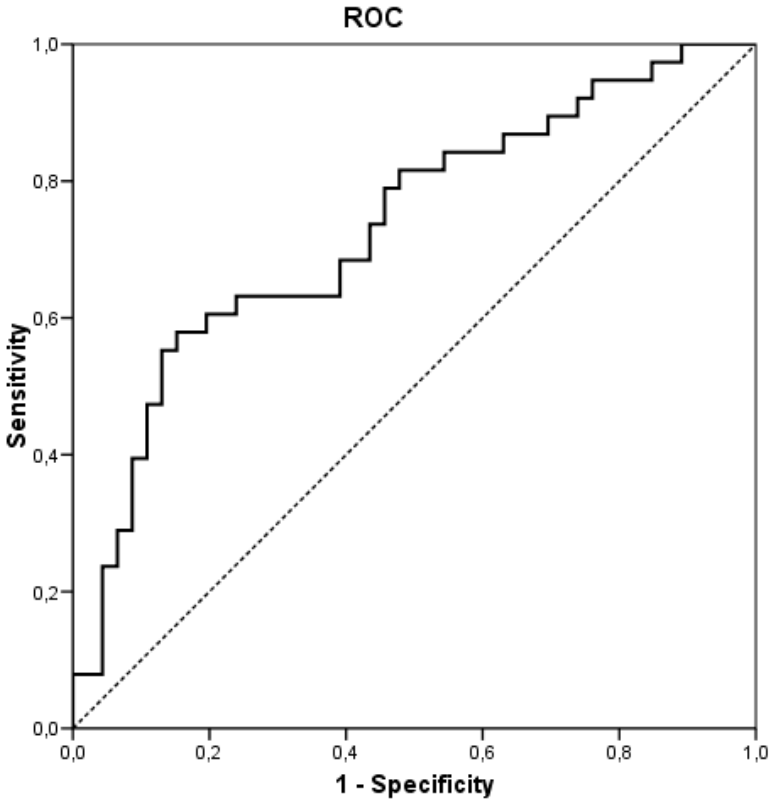

Figure 6. Roc curve analyzing cut-off value of R_2HG in all glioma patients

Analyzing only HGG patients, the optima cut-off value of R_2HG was 20 with which sensitivity and specific was 76% and 89%, respectively; its accuracy was 84, positive predictive value was 80% and negative predictive value was 86% (see figure 7 and Figure 8).

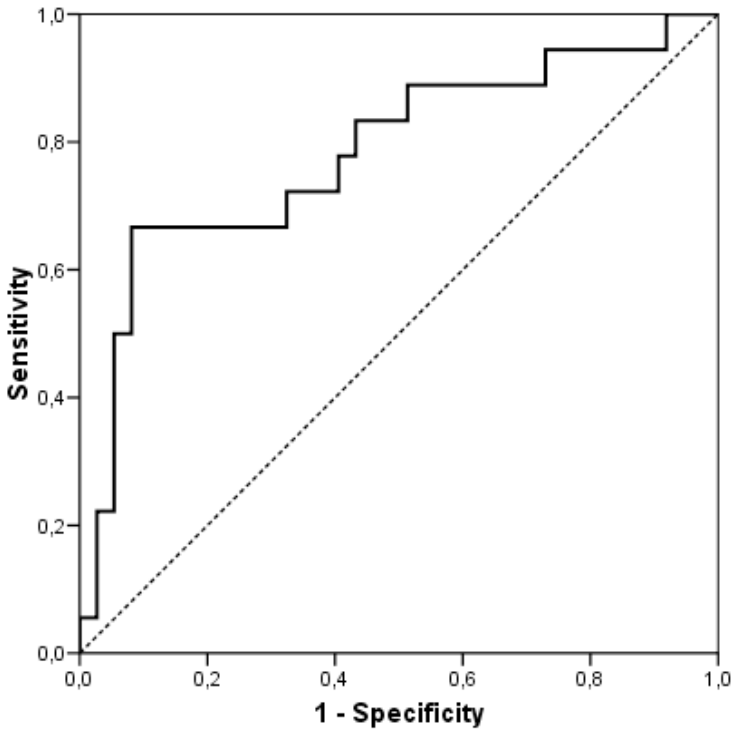

Figure 7. Roc curve analyzing cut-off value of R_2HG in high-grade glioma patients

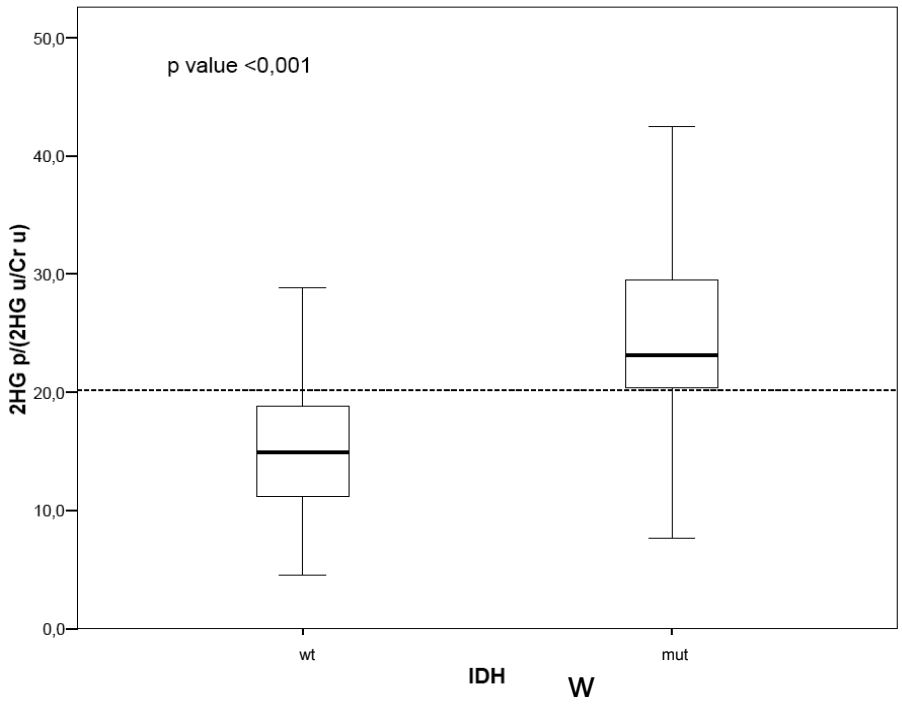

Figure 8. Levels of R_2HG in high-grade glioma patients with and without IDH mutation. The optimal cut-off value of R_2HG showed in dashed line.

Subsequently, we performed an analysis of correlation between the tumor size and R_2HG in HGG patients with IDH1 mutated (see Figure 9). We demonstrated that R_2HG levels are independent of the tumor size (Spearman R= 0.4; p= 0.8).

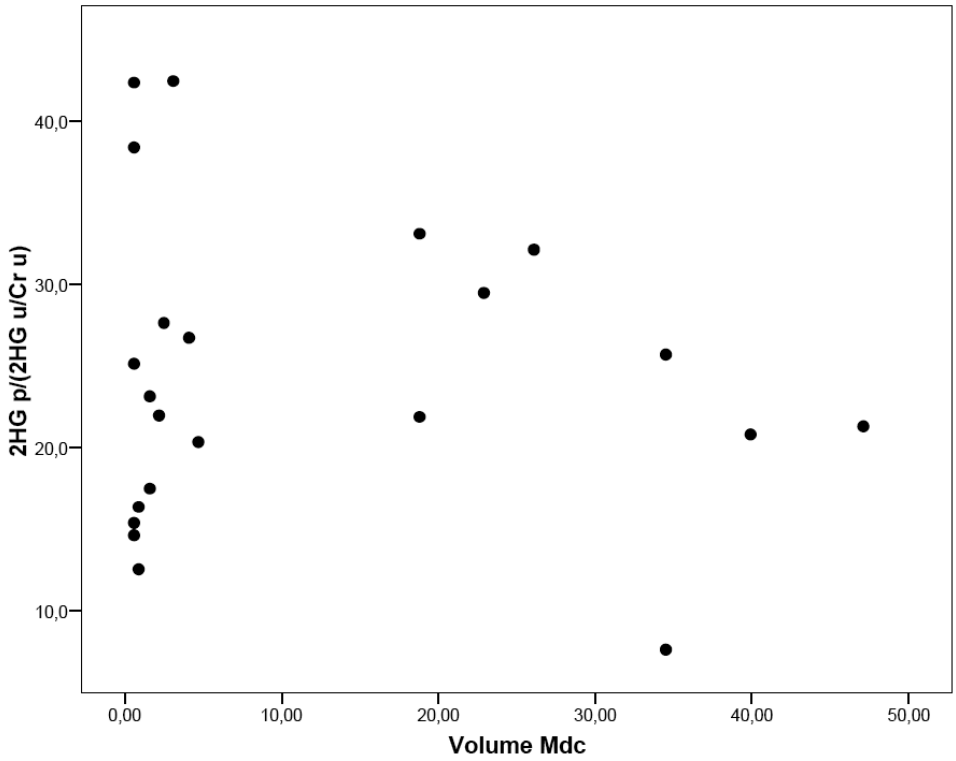

Figure 9. Correlation between R_2HG and the tumor size in high-grade patients with IDH1 mutated.

We investigated if there is an association between tumor grade and R_2HG in all glioma patients. We showed that R_2HG levels are independents of tumor grade; in

fact, there is no statistically significant difference of R_2HG among gliomas with different tumor grade (p= 0.4) (see Figure 10).

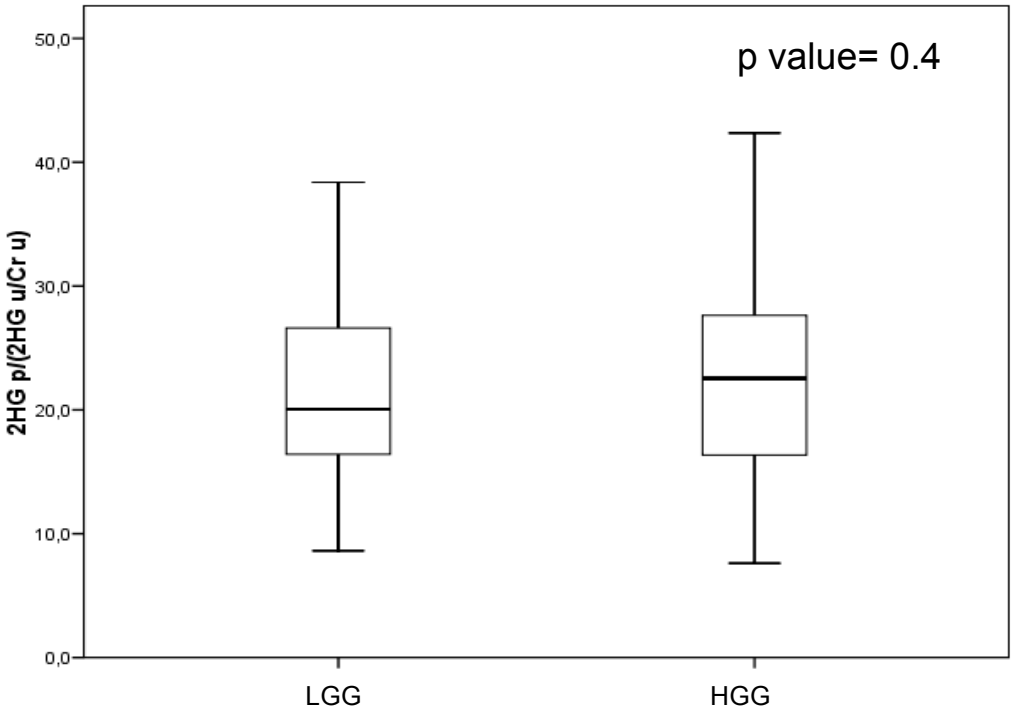

Figure 10. Analysis of R_2HG in LLG and HGG patients with IDH mutated. LGG= low grade glioma; HGG= high grade glioma.

Finally, we analyzed in a few patients the levels of R_2HG before and after the chemotherapeutic treatment; in particular, we performed a gadolinium-brain magnetic resonance imaging (MRI) before and after the treatment and correlated the R_2HG levels to the MRI response evaluated according to RANO criteria. A valid biomarker should increase in case of progression disease on MRI and should decrease in case of partial or complete response on MRI.

We analyzed 7 patients with HGG and 4 patients with LGG.

Among patients with HGG we had: 4 partial responses, 1 complete response and 2 progression diseases. In all cases of response we had a decrease of R_2HG, while in both the cases of progression disease the biomarker was increased (see Figure 11).

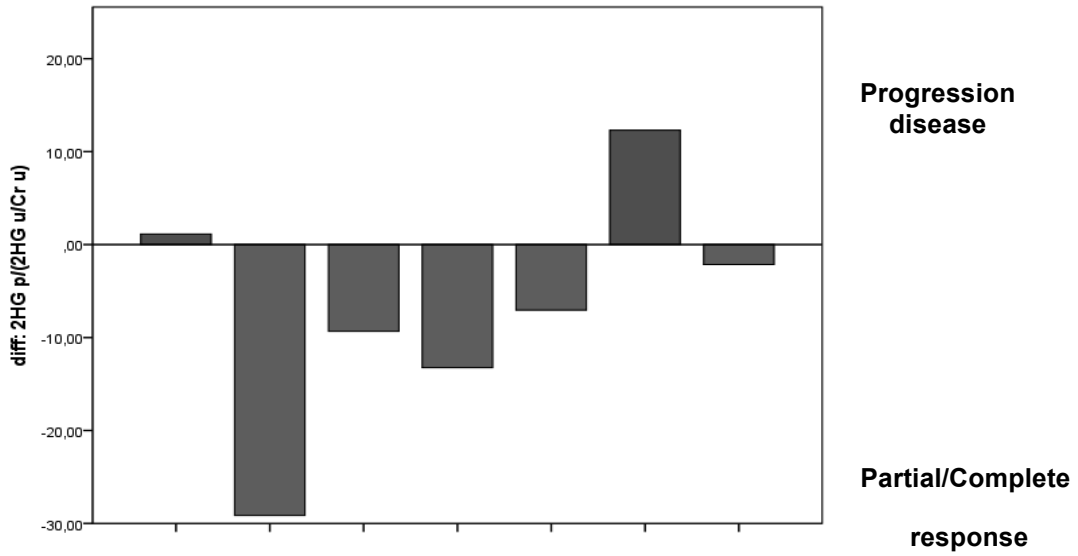

Figure 11. Difference of R_2HG value before and after the chemotherapeutic treatment and its correlation with response on brain-MRI in seven patients with high-grade gliomas

Among patients with LGG we had: 3 partial responses and 1 stable disease. Unlike the high-grade gliomas, in these patients there were discordance between response on MRI and levels of R_2HG; in fact, in one case of partial response the biomarker was increased while in the other two cases the biomarker was decreased; in the patient with stable disease the biomarker was increased slightly.

DISCUSSION

Gliomas comprise the second most common primary central nervous system neoplasms, behind meningiomas, and account for 80% of primary, malignant brain tumors (1).

Gliomas are classified by the resemblance of the neoplastic cells to normal glial types, and in the current WHO classification include those of astrocytic lineage, oligodendroglial lineage and those showing mixed lineage (oligoastrocytomas) (2). WHO classification of gliomas is based on a grading scheme from II to IV based on histomorphology, proliferation, and the presence of microvascular proliferation or necrosis; however, this classification has limitations; in particular, classification and grading of gliomas is accompanied by a considerable interobserver variation (23) and moreover, significant heterogeneity in clinical behavior among tumors with the same grade and clinical features is observed.

In the last years, it is becoming evident that gliomas can be separated into well characterized prognostic groups based on molecular profiling (24).

The recent discovery that genes encoding IDH1 and IDH2 can be mutated in gliomas represents one of the biggest success stories for cancer biology. The most frequent mutations affect codon 132 in IDH1 and 172 in IDH2. These mutations occur in 70-80% of grade II and III astrocytomas, oligodendrogliomas, oligoastrocytomas and secondary glioblastomas (25). Mutant IDH enzymes show a neomorphic enzymatic capacity to convert α-KG into 2HG, a small oncometabolite; in fact, 2HG inhibits α-KG binding to several histone demethylases leading to a widely aberrant histone modification profile, particularly histone tail methylation. 2HG also inhibits the TET

oncogene 1 and 2 hydroxymethylases; finally, 2HG can decrease levels of HIF-1 antagonist endostatin increasing VEGF (vascular endothelial growth factor) signaling (26).

Various studies demonstrated that gliomas of grade II-IV carrying IDH mutations have a better prognosis than IDH wild-type tumors (15, 27-29) and so, IDH mutation status should be added to current WHO classification. Moreover, enzymes defects are attractive molecule for targeted therapy such as restoring normal IDH function, replacing depleted α-KG or deplecing 2HG; and so, the presence of IDH mutations may have a predictive factor, as well. Most interestingly, a selective R132H-IDH1 inhibitor has recently been shown to specifically impair the growth of IDH1-mutant glioma cells and promote their differentiation (30).

The assessment of IDH status can be used as a diagnostic marker, as well. In fact, the presence of IDH mutation can recognize diffuse tumor infiltration of astrocytoma or oligodendroglioma, identify residual glioma cells in tissue with extensive post-therapy changes, distinguish diffuse tumor infiltration from reactive gliosis, distinguish diffuse astrocytomas as opposed to pilocytic astrocytoma (WHO grade I), distinguish astrocytic and oligodendroglial tumors from ependymomas, distinguish oligodendrogliomas from other glioneural tumors.

Currently available methods of detecting IDH mutations in gliomas are based on analysis of glioma tissue investigating either the altered structure of the protein, by monoclonal antibody against the common mutation IDH1 R132H, or the sequence of the IDH gene by gene sequencing. The sensitivity and specificity of monoclonal antibody for detecting IDH1 R132H reportedly approaches 100% (31) although the other rare mutations are not detected. While gene sequencing can identify all possible

mutations within the region amplified, the sensitivity of this method is about 20% of mutant sequences in a wild-type background, and so the mutant template must represent a relatively high proportion of the tissue to allow detection (32, 33).

Noteworthy, not all patients with gliomas can be amenable to biopsy because of their poor performance status or localization of the lesion in sensitive areas, such as the brainstem. An interesting non-invasive method to identify IDH status mutation was described by Sanson et al (34); they extracted small-size DNA (150-250 base pairs) from the plasma and IDH-R132H mutation was detected by a combination of coamplification at lower denaturation temperature and digital PCR; The IDH-R132H mutation was detected in 15 out of 25 plasma DNA mixtures (60%) from patients with mutated tumors and in none of the 14 patients with a non-mutated tumor. The specificity was 100% while the sensitivity was related to the tumor volume: 33% in non-enhancing tumors, 60% for enhancing volume $<10cm^3$, and 100% (6/6) for enhancing volume $\geq 10cm^3$. And so, this method can be used in gliomas with large contrast enhancement while other methods have to be used for low grade gliomas, such as the analysis of 2HG detection by magnetic resonance spectroscopy (35) with a sensitivity of 100% and a 26% of false positive rate in the detection of 2HG; in fact, 2HG is 100-fold increased in glioma cells with IDH mutations (21) and the non-invasive detection of increased 2HG levels may serve as a surrogate marker for various type of IDH mutations.. Since 2HG is a small molecule it seems possible that it could reach the systemic circulation and to be excreted by urine, as showed in patients with 2-hydroxyglutaric aciduria, a metabolic disease due to 2HG dehydrogenase gene mutations (36). And so, we evaluated whether 2HG levels in urine and plasma may correlate with IDH mutation status. As described by a prior study (37), we found no statistical difference in plasma concentration between

patients with and without IDH1 mutation. For the first time worldwide, we analyzed the 2HG levels in urine in patients with gliomas and surprisingly, we found that patients with IDH1 mutation have a lower 2HG concentration than patients lacking the mutation. IDH1 mutation generate D-enantiomer of 2HG and not the L isomer of 2HG (21); likely, these isomers may have a different time of excretion by urine resulting in a lower concentration of urinary 2HG in patients with IDH mutations because of excess accumulation of the enantiomer D-2HG. Noteworthy, the ratio between 2HG concentration in plasma and urine was significantly higher in patients with IDH1 mutation and especially, in patients with high-grade gliomas, probably reflecting a disruption in the blood-brain barrier. We found that the optimal cut-off value to discriminate the IDH mutation status in high-grade gliomas was 20 with an important sensitivity, specificity and accuracy; in particular, this cut-off has a high negative and positive predictive value (86% and 80%, respectively). On the other side, we did not find a correlation between tumor size or tumor grade and R_2HG levels.

Although we analyzed only 7 patients with high-grade glioma, we showed that detection of R_2HG may provide a method to follow up disease progression in these patients with IDH1 mutant tumor and to monitor treatment effects; however, a large prospective study should be planned to clarify this aspect.

In conclusion, the presence of IDH1 mutation represents a valid prognostic and diagnostic marker for glioma patients; moreover, in the future it might predict efficacy of therapies targeted against this alteration. So, the identification of a reliable surrogate biomarker of IDH1 mutation in accessible bodily fluids would be useful, in particular in patients who are not amenable to biopsy. We demonstrated that the ratio between 2HG levels in plasma and urine can be used as a biomarker to differentiate glioma patients with and without IDH mutation, especially in patients with high-grade

gliomas. Moreover, a larger samples need to be analyzed to investestigate this method in patients follow-up for recurrence detection and to monitor treatment efficacy.

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