



Deregulation of ion channel and transporter encoding genes in pediatric gliomas

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Brain tumors, including the majority gliomas, are the leading cause of cancer-related death in children. World Health Organization has divided pediatric brain tumors into different grades and, based upon cDNA microarray data identifying gene expression profiles (GEPs), it has become evident in the last decade that the various grades involve different types of genetic alterations. However, it is not known whether ion channel and transporter genes, intimately involved in brain functioning, are associated with such GEPs. We determined the GEPs in an available cohort of 10 pediatric brain tumors initially by comparing the data obtained from four primary tumor samples and corresponding short-term cultures. The correspondence between the two types of samples was statistically significant. We then performed bioinformatic analyses on those samples (a total of nine) which corresponded to tumors of glial origin, either tissues or cell cultures, depending on the best "RNA integrity number." We used R software to evaluate the genes which were differentially expressed (DE) in gliomas compared with normal brain. Applying a *p*-value below 0.01 and fold change ≥ 4 , led to identification of 2284 DE genes. Through a Functional Annotation Analysis (FAA) using the NIH-DAVID software, the DE genes turned out to be associated mainly with: immune/inflammatory response, cell proliferation and survival, cell adhesion and motility, neuronal phenotype, and ion transport. We have shown that GEPs of pediatric brain tumors can be studied using either primary tumor samples or short-term cultures with similar results. From FAA, we concluded that, among DE genes, pediatric gliomas show a strong deregulation of genes related to ion channels and transporters.

Keywords: pediatric gliomas, gene expression profiling, short term culture, ion channels and transporters, biological processes

INTRODUCTION

Brain tumors are the most common solid malignancy and the leading cause of cancer-related deaths in children (Pollack et al., 2006; Dubuc et al., 2010). Among these cancers, gliomas represent the vast majority, ranging from 56 to 70%, depending on the registry and the histological criteria used (Qaddoumi et al., 2009). The most common pediatric gliomas are astrocytomas and ependymomas. They are classified as low grade gliomas (LGGs) and high grade gliomas (HGGs; Qaddoumi et al., 2009). According to the World Health Organization (WHO), low grade astrocytomas and ependymomas include pilocytic astrocytomas (PAs) WHO grade I, subependymal giant cell astrocytomas (SEGAs) WHO grade I, pleomorphic xanthoastrocytomas (PXAs) WHO grade II, fibrillary astrocytomas (FAs) WHO grade II, ependymomas WHO grade II, subependymomas WHO grade I, and myxopapillary

ependymomas WHO grade I (Louis et al., 2007). On the other hand, the high grade astrocytoma and ependymoma group is represented mainly by anaplastic astrocytomas (AAs; WHO grade III), glioblastoma multiforme (GBM; WHO grade IV), and anaplastic ependymomas WHO grade III. In the group of low grade ependymomas, we also included the newly identified angiocentric glioma (AG), which is currently interpreted as predominantly juvenile WHO grade I ependymoma. GBM and AA are more frequent in adults (Qiu et al., 2008) and represent only 7% of all pediatric brain tumors (Rickert et al., 2001). Despite aggressive surgical resection and radiotherapy accompanied or followed by adjuvant chemotherapy, pHGGs prognosis remains poor (Massimino et al., 2010).

Gene mutations, copy number aberrations, structural rearrangements, or deregulation of the transcriptome have been

shown to contribute to the development of brain tumors and currently play an integral role in their classification (Mischel et al., 2004; Dubuc et al., 2010). Moreover, the definition of molecular pathways differently expressed in brain tumors can help in identifying putative therapeutic targets (Bredel et al., 2005; Faury et al., 2007; Dubuc et al., 2010). Toward this aim, numerous recent studies, mainly on adults, have employed highly sensitive molecular techniques, such as comparative genomic hybridization (Koschny et al., 2002; Wiltshire et al., 2004) and cDNA microarrays (de Bont et al., 2008; Dubuc et al., 2010). In particular, cDNA microarrays are enabling evaluation of genome-wide gene expression profiles (GEPs) of brain tumors. It has been shown that significant molecular heterogeneity exists within morphologically defined cancers (Rorive et al., 2006) and that the determination of GEPs in gliomas strongly affects prediction of survival (Freije et al., 2004). Nevertheless, clinically relevant molecular signatures have been identified among brain tumors. From these studies, it has emerged that LGGs are characterized by an “antimigratory gene profile,” which is progressively lost in HGGs (Rorive et al., 2006). The latter appear to be characterized more by deregulation of genetic pathways belonging to DNA repair, transcriptional regulation, cell division, cell proliferation, and survival (Vital et al., 2010). Integrated molecular genetic profiling of pediatric HGGs revealed key differences with adult HGGs (Paugh et al., 2010). Such molecular differences can in turn affect prognosis (Faury et al., 2007).

Ion channels and transporters are increasingly considered to bear pathophysiological relevance in tumor biology (Arcangeli et al., 2009; Arcangeli and Yuan, 2011). Although these proteins are inherent to brain (neuronal and glial) functioning and have been shown to be dysregulated in brain tumors (Preussat et al., 2003; Sontheimer, 2008; Cuddapah and Sontheimer, 2011; Arvind et al., 2012), only a few studies have specifically studied the genes encoding ion channels and transporters and their relevance in pediatric gliomas. Such small number of studies is due to the low incidence of the disease and the rarity of tissues appropriate for molecular characterization (Rorive et al., 2006; Paugh et al., 2010; Vital et al., 2010). Besides affecting the neoplastic progression, the ion channel and transporter complement of glioma cells affects how these cells alter the composition of the peritumor space, particularly the pH, $[K^+]$, and neurotransmitter levels. These factors likely contribute to cause tumor-related epileptic seizures, a very serious morbidity factor in brain cancer with a poorly understood pathophysiology (Shamji et al., 2009).

On the whole the importance of obtaining a full picture of the membrane transporter expression in brain cancer is clear.

In this study, we analyzed GEPs of a cohort of pediatric glioma (mainly LGG) samples. Due to the limited quantity and heterogeneity of the biological material available, we first confirmed that short-term culturing of the glioma samples would not alter their GEPs, while allowing to obtain higher amounts and better quality of RNA. Subsequent evaluation of differentially expressed (DE) genes was based on Functional Annotation Analysis (FAA).

MATERIALS AND METHODS

TISSUE COLLECTION

Surgical specimens were obtained from the Neurosurgery Unit, Meyer Children's University Hospital, Firenze, Italy. Informed

consent for the data collection was obtained from the parents, or the legal guardians. The study overall was approved by the local international review board of the Hospital. From each fresh surgical specimen a macroscopically representative fragment was selected, that was further cut into two corresponding pieces. One was further processed for histological analysis and diagnosis. An expert pathologist, AMB, determined the histological diagnosis using standard criteria (Buccoliero et al., 2002). The other piece was further subdivided under sterile conditions. The two pieces were immersed either in RNeasy lysis solution (Qiagen by Life Technologies, USA), for RNA extraction or in phosphate buffered saline (PBS), until minced for obtaining the primary culture. The cohort comprised five PAs (WHO grade 1), one AG (WHO grade 1), one ependymoma (E; WHO grade 2), one anaplastic oligodendroglioma (AO; WHO grade 3), one atypical meningioma (AM; WHO grade 2), and 1 GBM (WHO grade 4; **Table 1**). The AM was used only for the comparison of the GEPs of fresh tissue versus cell culture.

PRIMARY CELL CULTURES

The surgical fragment collected and maintained in PBS under sterile conditions was mechanically minced with sterile blades in 60 mm Petri dishes (Euroclone Ltd, Celbio, Italy) and transferred into 15 ml tubes (SARSTEDT S.R.L., Italy) containing Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12, Gibco-Invitrogen, Italy) and 0.25 mg/ml of type II collagenase. After incubating the minced tissue at 37°C for 5 min, cells were pelleted and resuspended in complete medium: DMEM/F-12 supplemented with 10% fetal bovine serum (Sigma, Italy) and antibiotics. Finally, the cell suspension was aliquoted into Petri dishes and maintained at 37°C in a humidified atmosphere containing 5% CO₂ in air. Medium was replaced, after washing twice with PBS every 2 days, during the whole culture period (15 days maximum). Cultures were used for RNA extraction no later than 2 weeks following plating, to avoid over growth of mesenchymal cells.

CDNA MICROARRAY: RNA EXTRACTION, LABELING, AND HYBRIDIZATION

Total RNA was isolated by Trizol reagent according to the manufacturer's protocol (Invitrogen S.R.L., Italy). RNA integrity was checked using the Agilent 2100 Bioanalyzer (Agilent Technologies Italia, Italy), evaluating the RNA integrity number (RIN) of each sample (Schroeder et al., 2006). High quality quantified RNA was purified on silica gel columns using the RNeasy MinElute Cleanup kit (QIAGEN S.p.A., Italy). The quality of the purified RNA was confirmed by checking on a NanoDrop spectrophotometer ND-1000 (NanoDrop Technologies, Germany). GEPs were determined using Agilent oligonucleotide microarray by one-color technology. About 500 ng of total RNA were used for each sample to generate cDNA probes. cDNA synthesis and labeling were performed by Agilent's Quick Amp Labeling Kit. Hybridization was performed on human whole genome 44,000 oligonucleotide microarrays using reagents and protocols provided by the manufacturer (Agilent Technologies, Manual Part Number G4140-90042). The Feature extraction software (Agilent, version 9.5), was used to quantify the

Table 1 | Characteristics of tumor samples included in the study.

Patient ID	Tumor type	WHO Grade	Location	Sex	Age (years)	Seizure	Fresh tissue sample RIN	Primary culture RIN
168	AM	II	Fronto-temporal region	F	13	No	8.0	9.3
186	PA	I	Cerebellum	M	7	No	7.7	9.2
192	PA	I	Cerebellum	M	6	No	2.5	8.4
224	AO	III	Right rolandic region	F	30	No	8.2	9.5
232	E	II	Right frontal region	F	11	No	7.8	N/A
234	GBM	IV	Giant, left hemisphere	F	8 months	No	6.9*	9.1
250	PA	I	Cerebellum	F	11	No	6.2	N/A
1001	AG	I	Left parieto-occipital region	F	10	Yes	2.4	9.0
0102	PA	I	Cerebellum	M	20	No	2.2	9.5
1002	PA	I	Cerebellum	M	10	No	7.3	8.1

All the samples included in the study are reported. AO (sample 224), PA (sample 186), AM (sample 168), and PA (sample 1001) were used for the GEP comparison between fresh tissue samples and their derived primary cultures (see **Figure 1**). The other analysis referred to glioma cohort were carried out only on samples with the best RIN value (in bold) and were performed in all the tumor types listed in the Table, excepted for the meningioma (AM, sample 168). Abbreviations: PA, pilocytic astrocytoma; AO, anaplastic oligodendroglioma; GBM, glioblastoma multiforme; E, ependymoma; AM, atypical meningioma; AG, angiocentric glioma; F, female; M, male; N/A, not available.

*For the determination of GEP of GBM sample the fresh tissue was used in the bioinformatic analyses because, despite the best RIN value, the RNA obtained from the short-term culture was not sufficient to perform microarray experiment.

intensity of fluorescent images. The FirstChoice[®] Human Brain Reference RNA (Ambion, Applied Biosystems, Life Technologies Corporation, USA) was used as a reference. The Ambion[®] FirstChoice[®] Human Brain Reference RNA is a high quality standard, suitable for human microarray analysis, pooled from different brain regions from 12 donors. The Reference RNA passes the strict quality control criteria for purity and integrity used for the MicroArray Quality Control (MAQC) project. FirstChoice[®] Human Brain Reference RNA is the same RNA (identical manufacturing lot) used for the MAQC project (MAQC Consortium, 2006).

Statistical analysis of microarray data

Data analysis was performed using R software version 2.11.0¹ by Bioconductor Packages² using row images (dat files), as obtained from the Feature extraction analysis (see above). Data were normalized using “scale” normalization (Smyth and Speed, 2003) as implemented in “limma” package, made available on Bioconductor (see text footnote 2).

Comparison between fresh samples and primary cultures

The log₂ normalized values were used to evaluate differences in gene expression between fresh tissue samples and their derived primary cultures. Four different brain tumor samples of different histological types (including a non-glial tumor, e.g., one meningioma), and their corresponding short-term cultures, were analyzed: PA (sample 186), AO (sample 224), AM (sample 168), and PA (sample 1002). It is worth noting that when multiple arrays are being compared to each other, the raw data from all chips should be normalized together. A normalization between arrays, like scale normalization, assumes a common distribution

for datasets. Our collection of samples is however heterogeneous, as it combines different patients and distinct pathologies. As such, it clearly displays a degree of intrinsic variability that should be carefully evaluated. On the other hand, in the following analysis, we were mainly interested in detecting those biological patterns that appear to be consistently altered in all recorded dataset, beyond the specific peculiarities that make each sample inherently different. Moreover, it can be argued that relatively few genes are DE across the different experiments, an *a priori* ansatz that one can subsequently validated. Inspired by this rationale, we proceeded with the aforementioned normalization strategy and checked *a posteriori* the correlation coefficients among scrutinized dataset. Interestingly, the correlation coefficients between the expression values obtained in the fresh tissue samples and the corresponding measures relative to the cell culture resulted in: PA (sample 186), 0.88; AO, 0.86; AM, 0.94; and PA (sample 1002), 0.90. The correlation coefficients among all other dataset returned values close to 0.8, so confirming on the whole our former assumption. In particular, cultured samples appear to preserve a large part of the biological information as contained in their original fresh homologs.

Analysis of DE genes

To perform the differential expression analysis, aimed to identify deregulated genes in gliomas, we considered the log₂ ratio of intensity values of each gene, respect to the corresponding intensity value in the control sample (normal brain). This analysis was therefore applied to nine samples, either fresh or culture, depending on the best RIN (see **Table 1**).

A One Sample *t*-test was applied to the log-ratios and a *p*-value was calculated for each gene. A Bonferroni procedure was applied to take into account the multiple testing correction. To pick out the DE genes we considered a threshold of 0.01 on the corrected *p*-value, plus an average cut off of fourfold changes.

¹<http://www.r-project.org/>

²<http://www.bioconductor.org/>

Functional annotation analysis

Functional annotation analysis was performed using NIH-DAVID software (version 6.7)³. Our aim was to find the most relevant Gene Ontology (GO) terms associated with DE genes. For this purpose, we used the parameter GOTERM_BP_FAT, and the significance *p*-value threshold was set <0.01, with Bonferroni multiple testing correction.

RESULTS

EVALUATION OF DIFFERENCES IN GENE EXPRESSION BETWEEN FRESH TISSUE SAMPLES AND THEIR DERIVED SHORT-TERM CULTURES

We first analyzed the GEPs of four fresh pediatric brain tumor samples of different histogenesis (see Materials and Methods) and their corresponding short-term culture (15 days). Thus, we determined whether the establishment and short-term maintenance of the cell culture would alter the GEP of the primary tissue sample. In **Figure 1**, the (normalized) gene expression values associated with the fresh tumor samples are plotted against the data obtained from the corresponding short-term cultures. Clearly, the two sets of data are positively correlated. This implied that the short-term culturing preserved a large portion of the original GEPs. On the other hand, and beyond the qualitative assessment, a dispersion around the idealized linear distribution was apparent (**Figure 1**). The latter observation reflected the inherent variability of the scrutinized samples, mainly due to the histopathological differences of tumors analyzed. In order to evaluate the data quantitatively, we calculated the correlation coefficient values between fresh tissue

samples and the corresponding culture, applying the assumptions and the procedure described in Section “Materials and Methods.” The values thus obtained were: A: 0.88, B: 0.86, C: 0.94, and D: 0.90. Based on the above, we argued that cultured samples preserve a substantial degree of similarity with their fresh analogs. We concluded, therefore, that either primary tissue samples or their corresponding short-term cultures, could be used to perform cDNA microarray analyses. This allowed the use of either short-term cultures or primary tissue samples depending on best RIN value. On these bases, we performed cDNA microarray analyses of DE genes in the available cohort of pediatric gliomas (**Table 1**).

ANALYSIS OF DIFFERENTIALLY EXPRESSED GENES OF GLIOMAS COMPARED TO NORMAL BRAIN

We determined the GEPs of nine gliomas, mainly LGGs (PAs, AG, AO, and E) and a GBM. Since the analysis mainly focused on glial-derived tumor, the AM sample was omitted. The Ambion® FirstChoice® Human Brain Reference RNA (Applied Biosystems, Life Technologies Corporation, USA), which derives from the pooling of 12 different donors and several brain regions, was used as the normal control reference. Although the tumor analyzed had different sites of presentation, we used a “whole brain” control sample, as previously described by Godard et al. (2003), to ensure a reproducible, tissue-specific reference. The expression profile of each gene was evaluated as detailed in Section “Materials and Methods.” In particular, a gene was assumed to be DE when the corrected *p*-value was lower than 0.01 and the fold change was ≥ 4 . We obtained 2284 probes matching such requisites. The 2284 DE genes are shown in a cluster diagram in **Figure 2** and are listed in Table S1 in Supplementary

³<http://david.abcc.ncifcrf.gov/>

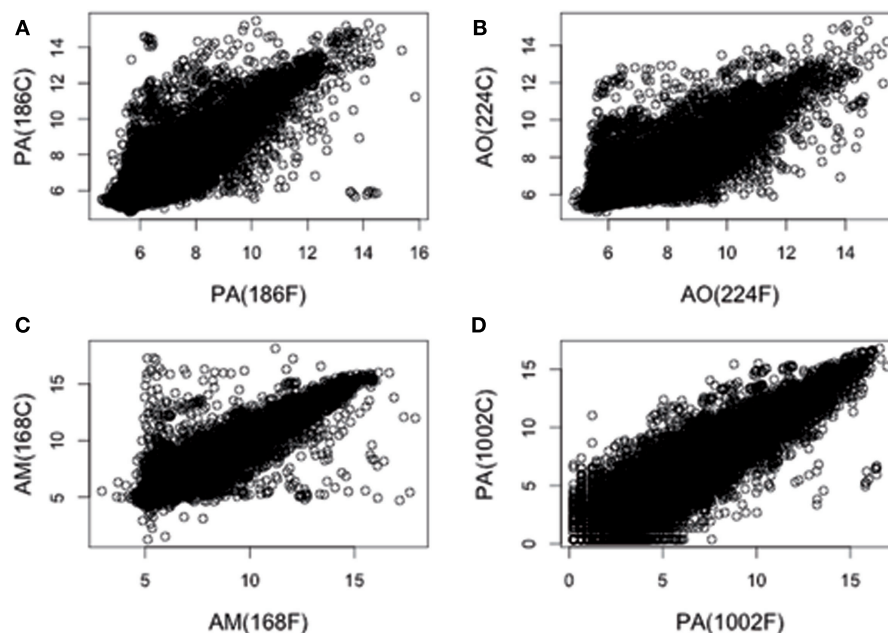
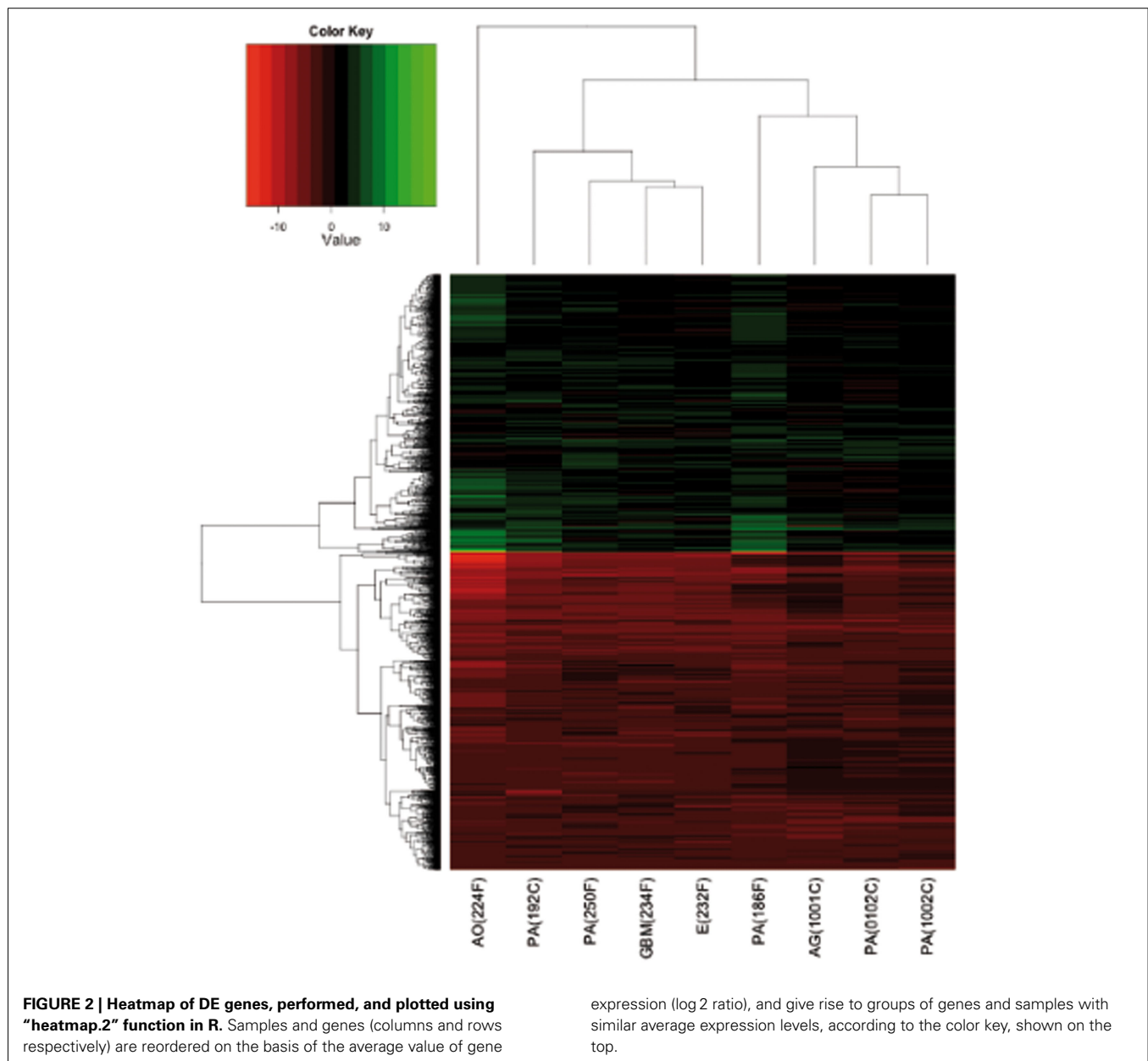


FIGURE 1 | Comparison of GEPs between fresh tissue samples and their derived primary cultures. The log₂ normalized values for each gene of fresh tissue pediatric brain tumors versus its primary culture are matched. In (A–D) are represented PA (sample 186), AO (sample 224), AM (sample 168), and PA

(sample 1002) samples, respectively. The degree of similarity of each sample respect to its primary culture has been quantitatively explored by calculating the associated correlation coefficients. Average Pearson’s correlation coefficients were: (A), 0.88; (B), 0.86; (C), 0.94, and (D), 0.90.



Material. About 1219 out of the 2284 DE genes (53%) were downregulated, whilst 1065 DE genes (47%) were upregulated (Figure 2).

FUNCTIONAL ANNOTATION ANALYSIS OF THE GLIOMA SAMPLES COHORT

We next performed FAA on the microarray data of DE genes, based on GO terms enrichment, using the NIH-DAVID software. It emerged that the DE genes are significantly associated to 43 groups (called “terms”) of potential functional distinction (Table S2 in Supplementary Material). This type of analysis (Huang et al., 2009) identifies the most altered biological processes, regardless their up or down expression. Hence, we performed a second FAA, taking into account separately those genes that are up or downregulated.

Upregulated genes

The FAA on the DE genes that are overexpressed led to 61 enriched terms (Table 2; Table S3 in Supplementary Material). The majority of them belong to three main biological processes that can be summarized as follows: immune/inflammatory response, cell proliferation and survival, cellular adhesion and motility. Table 2 shows the upregulated terms divided into the three main biological processes identified. Some representative genes, with their median expression values (reported as fold change), are also reported.

Downregulated genes

Functional annotation analysis of these genes instead revealed 12 terms. Applying the same criteria of selection as in the FAA of upregulated genes, we found that they were mostly related to two main biological processes: neuronal phenotype and ion transport

Table 2 | Statistically significant biological processes, associated to the upregulated genes only, represented by DE GO terms in our cohort of glioma samples.

Biological processes	GO terms	Representative genes	Fold change
Immune/inflammatory response	GO:0006955 ~ immune response	C1QA	2.25
	GO:0009611 ~ response to wounding	ANXA1	3.61
	GO:0006952 ~ defense response	ANXA5	2.99
	GO:0006954 ~ inflammatory response	CTGF	6.20
	GO:0019882 ~ antigen processing and presentation	CD163	3.12
	GO:0042060 ~ wound healing	NOTCH2	2.21
	GO:0048002 ~ antigen processing and presentation of peptide antigen	CD44	3.13
	GO:0010033 ~ response to organic substance	STAT1	2.78
	GO:0002684 ~ positive regulation of immune system process	MYC	2.75
	GO:0046649 ~ lymphocyte activation	COL1A1	6.72
	GO:0002460 ~ adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains	IFI30	4.69
	GO:0002250 ~ adaptive immune response	CDKN1A	2.26
	GO:0042110 ~ T cell activation	CDKN2A	2.91
	GO:0045321 ~ leukocyte activation	ERBB2	2.48
	GO:0002504 ~ antigen processing and presentation of peptide or polysaccharide antigen via MHC class II	HBEGF	3.13
	GO:0045087 ~ innate immune response	FN1	4.86
	GO:0002694 ~ regulation of leukocyte activation		
	GO:0002252 ~ immune effector process		
	GO:0050778 ~ positive regulation of immune response		
	GO:0051249 ~ regulation of lymphocyte activation		
GO:0006968 ~ cellular defense response			
GO:0002449 ~ lymphocyte mediated immunity			
Cell proliferation and survival	GO:0043067 ~ regulation of programmed cell death	KCNMA1 (variant 2)	2.53
	GO:0042127 ~ regulation of cell proliferation	ANXA5	2.99
	GO:0010941 ~ regulation of cell death	ANXA1	3.61
	GO:0042981 ~ regulation of apoptosis	MMP9	6.35
	GO:0043065 ~ positive regulation of apoptosis	CD44	3.13
	GO:0043068 ~ positive regulation of programmed cell death	NOTCH2	2.21
	GO:0010942 ~ positive regulation of cell death	IFI30	4.69
	GO:0006917 ~ induction of apoptosis	STAT1	2.78
	GO:0012502 ~ induction of programmed cell death	MYC	2.75
	GO:0008285 ~ negative regulation of cell proliferation	CDKN1A	2.26
	GO:0006915 ~ apoptosis	CDKN2A	2.91
	GO:0008219 ~ cell death	ERBB2	2.48
	GO:0008284 ~ positive regulation of cell proliferation	HBEGF	3.13
	GO:0016265 ~ death	PIM1	2.76
	GO:0012501 ~ programmed cell death		
	GO:0043066 ~ negative regulation of apoptosis		
	GO:0006916 ~ anti-apoptosis		
	GO:0043069 ~ negative regulation of programmed cell death		
GO:0060548 ~ negative regulation of cell death			
Cellular adhesion and motility	GO:0006928 ~ cell motion	ANXA1	3.61
	GO:0016477 ~ cell migration	CTGF	6.20
	GO:0051674 ~ localization of cell	CD44	3.13
	GO:0048870 ~ cell motility	MMP9	6.35
	GO:0007155 ~ cell adhesion	COL1A1	6.72
	GO:0022610 ~ biological adhesion	TGFBI	6.98

(Continued)

Table 2 | Continued

Biological processes	GO terms	Representative genes	Fold change
	GO:0030198 ~ extracellular matrix organization	ANXA2P1	4.99
	GO:0051270 ~ regulation of cell motion	ANXA2	5.02
	GO:0030334 ~ regulation of cell migration	ERBB2	2.48
	GO:0006935 ~ chemotaxis	HBEGF	3.13
	GO:0042330 ~ taxis	FN1	4.86
	GO:0040012 ~ regulation of locomotion		

Some representative genes are reported with the relative fold change.

(Table 3; Table S4 in Supplementary Material). Table 3 shows the downregulated terms divided into the two main biological processes identified. Some representative genes, with their median expression values (reported as fold change), are also reported.

ANALYSIS OF DE GENES ASSOCIATED TO THE TRANSPORTER CLASSIFICATION DATABASE

To further deepen the study of ion transport process, we performed a more focused analysis selecting, among the DE genes, the probes associated to ion channels, and transporters according to the readily available Transporter Classification Database for ion channels and transporters⁴. We thus identified 67 DE genes (Figure 3; Table S5 in Supplementary Material). Such genes included two main groups: those coding for transporters ($n = 45$; 67%) and those coding for ion channels ($n = 22$; 33%). Most of these genes encode solute carriers (SLC, $n = 25$), whereas other categories include potassium channels ($n = 16$), ATPases ($n = 8$), ATP-binding cassettes (ABC, $n = 7$), calcium channels ($n = 5$), annexins (ANXA, $n = 4$), and two sodium channel beta subunits. Thirty-three percentage of the 67 genes belonging to ion channels and transporters are upregulated and they are mainly transporters. On the other hand, among the downregulated genes, many (48%) belongs to ion channel encoding genes.

DISCUSSION

We have applied a method, previously developed for adult gliomas (Masi et al., 2005), to obtain short-term cultures of pediatric gliomas. This method proved to be suitable for cDNA microarray studies of pediatric brain tumors, where the availability of fresh primary samples is limited by surgical procedures and diagnostic necessities, as well as possible ethical constraints. Moreover, establishing tissue cultures providing a “pure” population of proliferating tumor cells makes it unnecessary to selectively isolate and capture malignant glial cells, for further analyses. Finally, the primary culture samples we analyzed often showed better RIN values (see Table 1), hence strongly influencing the decision whether to include or not a patient into a study. Indeed, when we plotted and calculated the correlation coefficients of the gene expression values associated with the fresh tumor samples against the data obtained from the corresponding short-term cultures, the two sets of data turned out to be positively correlated. This implied that the short-term culturing preserved a

large portion of the original GEPs. This method, when applied routinely, could overcome possible molecular degradation and contamination by non-cancerous cells (although some differentiation or selection of unwanted cellular phenotypes could not be ruled out), which often biases molecular studies in primary tumor samples.

The short-term culture system allowed the molecular characterization of the tumors, and could be extended to further functional studies. In the present study we determined the GEPs of nine gliomas (mainly LGGs/PAs), a small number of samples, although compatible to those reported in recent studies (Wong et al., 2005; Faury et al., 2007; Kasuga et al., 2008). Almost half of the DE genes were upregulated, while the others were downregulated. The further FAA led us to identify terms of the DE genes in our samples. Besides being obtained with different biomolecular and analytical procedures, the genes we identified as DE belonged to terms almost identical to those reported in Wong et al. (2005) and Faury et al. (2007). This further stress the validity of the methods we applied in the present study.

In fact, that the terms comprising the overexpressed DE genes were mainly those concerned with immune/inflammatory response, control of proliferation/survival, and regulation of cell adhesion and migration.

In particular, the genes belonging to the immune/inflammatory response are represented by human leukocyte antigen (*HLA*, e.g., *MHC*) genes, mainly *HLA-D* genes. Since it is known that neurons under normal conditions inhibit MHC expression in glial cells (Tontsch and Rott, 1993), the overexpression of MHC genes in the tumor glia could be the consequence of neuronal damage leading to alteration of neuroglial contacts accompanied by the difficulty for neurites to establish contact with cells placed within the tumor mass. Alternatively, tumor astrocytes could become APC-like cells and thus upregulate their MHC II class expression as suggested earlier (Vidovic et al., 1990; Nair et al., 2008).

Among the upregulated genes, many growth factors (GFs) are comprised (Table 2; Table S1 in Supplementary Material). Interestingly, while GFs such as *TGFBI*, *CTGF*, *VEGF*, *HBEGF*, *ERBB2*, platelet derived GFs, insulin like GFs turned out to be overexpressed, fibroblast GFs, and their receptors were downregulated. This confirmed the accuracy of our short-term culture system, ruling out any contamination by mesenchymal cells. The deregulation of genes involved in proliferation and survival also involved cell cycle regulating genes, such as *CDKN1A* and *CDKN2A*. *CDKN1A*

⁴http://www.tcdb.org/hgnc_explore.php

Table 3 | Statistically significant biological processes, associated to the downregulated genes only, represented by DE GO terms in our cohort of glioma samples.

Biological processes	GO terms	Representative genes	Fold change
Neuronal phenotype	GO:0019226 ~ transmission of nerve impulse	KCNMA1 (variant 1)	-2.82
	GO:0007268 ~ synaptic transmission	SLC1A2	-2.84
	GO:0030182 ~ neuron differentiation	SCN2B	-3.01
	GO:0031644 ~ regulation of neurological system process	SCN4B	-2.82
	GO:0051969 ~ regulation of transmission of nerve impulse	NEUROD2	-7.74
	GO:0050804 ~ regulation of synaptic transmission	STMN2	-8.49
	GO:0048666 ~ neuron development		
Ion transport	GO:0030001 ~ metal ion transport	KCNMA1 (variant 1)	-2.82
	GO:0006811 ~ ion transport	CACNA2D2	-2.27
	GO:0006812 ~ cation transport	ATP1B1	-4.60
		SCN2B	-3.01
		SCN4B	-2.82
		SLC30A3	-4.08
		ATP6V1G2	-4.49
		ATP6V0C	-2.11
		SLC26A1	-3.15
		SLCO4A1	-2.45
	SLC4A3	-2.52	

Some representative genes are reported with the corresponding fold change.

plays also a role in cell protection from apoptosis. Indeed, several other genes involved in different apoptotic pathways, presented altered regulation in our samples. For example, members of BCL families, regulatory genes such as *MYC*, *STAT1*, and *NOTCH2* were all overexpressed in our tumor samples.

Moreover, many genes related to the extracellular matrix (ECM) turned out to be overexpressed in our samples. Among them, the matrix metalloproteinase (MPPs) MMP9 (6.35, as reported in **Table 2**; Table S1 in Supplementary Material) was highly overexpressed. MMPs upregulation followed the overexpression of genes that encode for ECM constituents such as collagens and fibronectin.

Significant downregulation of genes encoding neuronal phenotype and ion channels and transporters was apparent in the pediatric glioma cohort we studied. An overall downregulation of genes belonging to the “neuronal phenotype” could be expected, since we compared glial origin tumors with normal brain tissue. Nevertheless, the deregulation of ion channels and transporters merits more attention. In particular, it is interesting to note that most of the downregulated ion channel genes were voltage-gated potassium and calcium channels. This would suggest deregulation of glial cell excitability, as previously indicated (Verkhatsky and Steinhäuser, 2000; Sontheimer, 2008). Only two potassium channel encoding genes were upregulated, *KCNQ1* (Barhanin et al., 1996) and *KCNMA1*, encoding the large conductance calcium-activated potassium channel, *KCa1.1*. *KCa1.1* was upregulated in various type of tumors, mainly of glial origin (D’Amico et al., 2012). In the present cohort of pediatric gliomas, only the variant 2 isoform was upregulated, while the variant 1 isoform was downregulated. Interestingly, also *CACNA2D2*, encoding a calcium channel, was found to be downregulated, in agreement

with Carboni et al. (2003) who identified *CACNA2D2* as a putative tumor suppressor gene. Finally, the alteration in the expression level of *SCN2B* and *SCN4B*, encoding two sodium channel subunit beta, further stresses the relevance of sodium channels in gliomas, as recently reported by Joshi et al. (2011). Normal expression of K^+ channels in astrocytes maintains an hyperpolarized potential which regulates buffering of extracellular K^+ , pH, and glutamate (Djukic et al., 2007; Olsen and Sontheimer, 2008). Our results show that generalized downregulation of K^+ channels occurs in gliomas, in agreement with previous studies on specific channel types (e.g., Olsen and Sontheimer, 2008). The ensuing alteration of the peritumor space composition is likely enhanced by the concomitant decrease in the expression of electrogenic glutamate transporters (De Groot and Sontheimer, 2011) and several transporters implicated in pH regulation, such as the sodium bicarbonate cotransporters (e.g., Table S5 in Supplementary Material). These effects may contribute to explain the proneness to epileptic seizure development displayed by LGG patients, as higher extracellular K^+ , lower pH, and increased glutamate levels tend to produce neuronal hyperexcitability (Buckingham et al., 2011).

While downregulation of ion channels may decrease the functioning of glial cells (Verkhatsky and Steinhäuser, 2000), deregulation of gene encoding transporters might have more complex consequences, contributing to multidrug resistance (Carboni et al., 2003; Lee et al., 2005; Chen et al., 2006), and/or enhanced cytotoxicity (Wakaumi et al., 2005; Blair et al., 2011). Moreover, the downregulation of genes encoding ion transporters, such as V-ATPase genes and *SLC4A3* and *SLC26A1* could strongly alter the pH homeostasis (Casey et al., 2009). Interestingly, the genes encoding for the SLC and ABC transporters

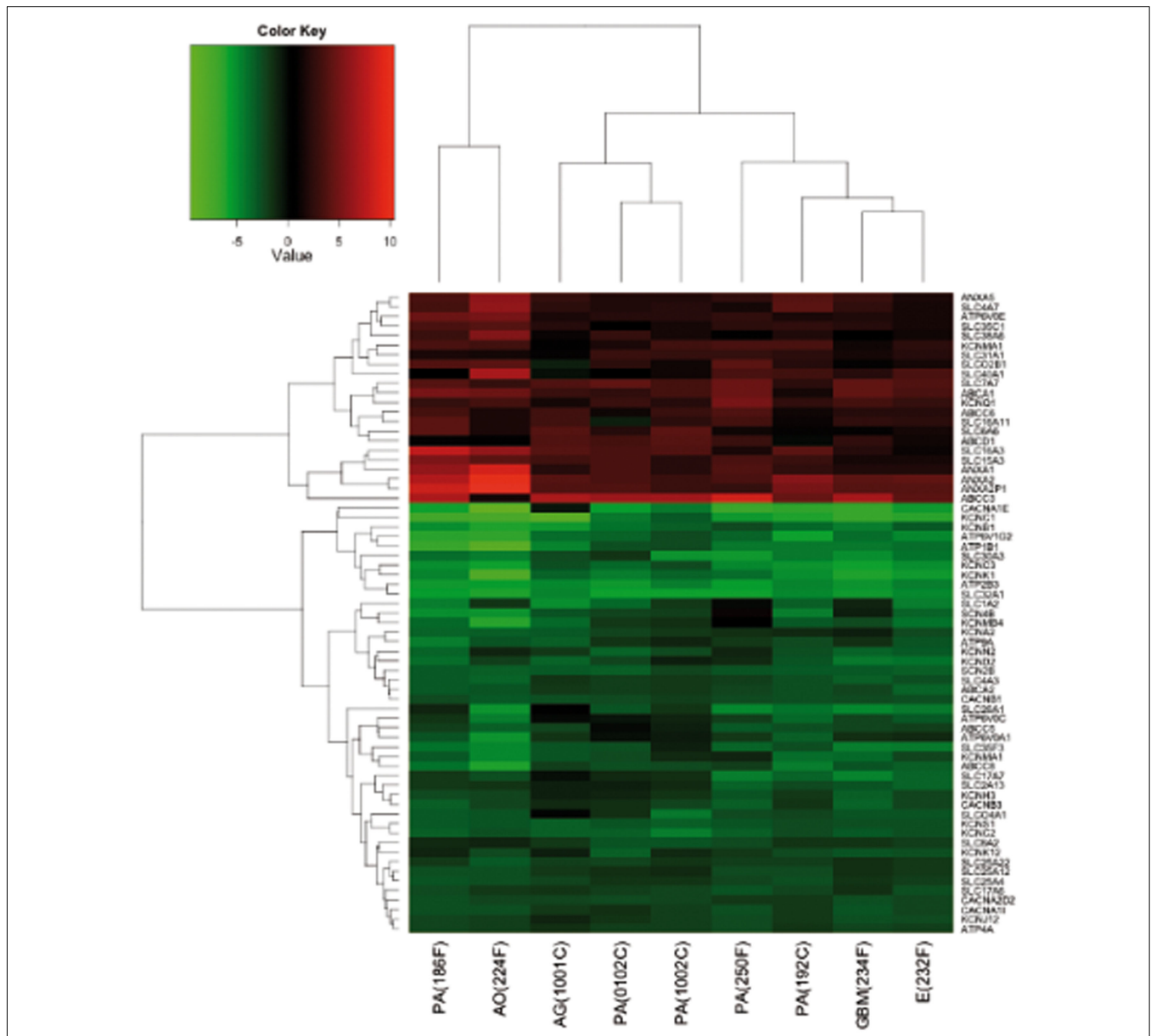


FIGURE 3 | Heatmap of DE genes associated to transporters and ion channels, performed, and plotted using “heatmap.2” function in R. Samples and genes (columns and rows respectively) are reordered on the

basis of the average value of gene expression (log₂ ratio), and give rise to groups of genes and samples with similar average expression levels, according to the color key on the top.

were almost equally distributed between the up and the down-regulated genes, whereas the ANXAs were upregulated and the ATPases were downregulated (except for the *ATP6V0E* transcript). The gene encoding *ABCC6*, which is involved in multidrug resistance, is one of the most strongly upregulated genes. Another transporter that merit attention is *SLC1A2*. *SLC1A2* expression tends to decrease glioma cell proliferation (Krona, 2006), and we assume its downregulation could contribute to increase proliferation in our cohort of samples. Moreover, because *SCL1A2* is the main responsible for clearing extracellular glutamate during excitatory synaptic activation in the central nervous system, downregulation of *SLC1A2* may be a further contributor

to seizure development (Simantov et al., 1999), as discussed above.

On the whole, determining the GEPs of individual patients could significantly affect the available therapeutic choices.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Pediatric_Oncology/10.3389/fonc.2012.00053/abstract

Table S1 | Gene differentially expressed (DE) in gliomas compared to normal brain. About 2284 DE genes identified by One Sample *t*-test with a *p*-value < 0.01, fold change ≥ 4 , and Bonferroni correction.

Table S2 | Functional annotation analyses (FAA) from all DE genes. FAA performed using NIH-DAVID software (version 6.7, <http://david.abcc.ncifcrf.gov/>) on all the DE genes reported in Table S1 in Supplementary Material. We used the parameter GOTERM_BP_FAT, with the significance *p*-value threshold set <0.01, with Bonferroni correction.

Table S3 | Functional annotation analysis from differentially overexpressed genes. FAA performed using NIH-DAVID software (version 6.7, <http://david.abcc.ncifcrf.gov/>) on the differentially overexpressed genes reported in Table S1 in Supplementary Material. We used the parameter GOTERM_BP_FAT, with the significance *p*-value threshold set <0.01, with Bonferroni correction.

Table S4 | Functional annotation analysis from differentially underexpressed genes. FAA performed using NIH-DAVID software (version 6.7, <http://david.abcc.ncifcrf.gov/>) on the differentially underexpressed genes reported in Table S1 in Supplementary Material. We used the parameter GOTERM_BP_FAT, with the significance *p*-value threshold set <0.01, with Bonferroni correction.

Table S5 | Differentially expressed genes associated to ion channels and transporters. Sixty-seven DE genes, associated to ion channels and transporters, identified by One Sample *t*-test with a *p*-value < 0.01, fold change ≥ 4 , and Bonferroni correction.

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