Gene Expression Profile of Glioblastoma Peritumoral Tissue: An Ex Vivo Study

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Abstract

The gene expression pattern of glioblastoma (GBM) is well documented but the expression profile of brain adjacent to tumor is not yet analysed. This may help to understand the oncogenic pathway of GBM development. We have established the genome-wide expression profiles of samples isolated from GBM tumor mass, white matter adjacent to tumor (apparently free of tumor cells), and white matter controls by using the Affymetrix HG-U133 arrays. Array-CGH (aCGH) was also performed to detect genomic alterations. Among genes dysregulated in peritumoral white matter, 15 were over-expressed, while 42 were down-regulated when compared to white matter controls. A similar expression profile was detected in GBM cells. Growth, proliferation and cell motility/adhesion-associated genes were up-regulated while genes involved in neurogenesis were down-regulated. Furthermore, several tumor suppressor genes along with the KLRC1 (a member of natural killer receptor) were also down-regulated in the peritumoral brain tissue. Several mosaic genomic lesions were detected by aCGH, mostly in tumor samples and several GBM-associated mosaic genomic lesions were also present in the peritumoral brain tissue, with a similar mosaicism pattern. Our data could be explained by a dilution of genes expressed from tumor cells infiltrating the peritumour tissue. Alternatively, these findings could be substantiated by a relevant amount of “apparently normal” cells presenting a gene profile compatible with a precancerous state or even “quiescent” cancer cells. Otherwise, the recurrent tumor may arise from both infiltrating tumor cells and from an interaction and recruitment of apparently normal cells in the peritumor tissue by infiltrating tumor cells.

Introduction

Glioblastoma (GBM) is the most common malignant tumor of the brain. GBM rapidly proliferates and invades the central nervous system. Due to its invasive characteristics, the prognosis of GBM patients is very poor, despite of the treatment that currently consists of surgical resection followed by radiotherapy plus concomitant and adjuvant temozolomide [1]. Targeted therapies have been introduced, based on information obtained from molecular studies of the tumor tissue (usually shown at MRI as an enhanced lesion, ET) [2]. However, no clear survival benefit has been demonstrated, probably because tumor tissue represents the last step of tumorigenesis, involving some alterations allowing tumor-cells to survive. Since recurrence in peritumoral tissue occurs in about 95% of patients [3], getting a deeper insight into the biology of the brain adjacent to-tumor (BAT) is of great interest. It has been demonstrated that the expression of a series of elements and amino-acids is altered in the BAT [4,5,6,7,8]. It has been shown that even in the absence of tumor cells, kinases involved in cell proliferation, migration and apoptosis are expressed in BAT together with molecules linked to stemness, invasion and neo-angiogenesis, which might indicate that BAT is undergoing transformation [9,10,11]. To support this hypothesis, we carried out a gene expression profile and a genomic analysis of BAT to detect alterations that may indicate the appearance of neoplastic features. To achieve our aim, we have compared the gene expression profile of tissue samples from ET, BAT, and normal white matter (CTRL) and have analyzed the BAT with an Array-CGH to detect genomic alterations.

Methods

Patients and Specimens

From January 2006 to December 2007, 60 adults were operated for primary GBM at our Institute. We selected 11 patients with tumor location far from eloquent areas. Among them, 5 patients

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did not present tumor cells in BAT and were included in the study. Tumor was removed with wide tumor-free resection margins of 1–2 cm. One part of the ET (without areas of necrosis) was immediately fixed in 10% neutral buffered formalin for histological analysis and the second one was immediately frozen on dry ice for molecular analysis. BAT specimens were obtained using a sampling-grid (Figure 1A). CTRL specimens were derived from 4 patients at the same ages operated for deep cavernomas with radiological signs of recent bleeding. These samples were used as controls in molecular analyses.

**Ethics statement.** All patients provided written consent to use their specimens for research purposes, none of them was identifiable. The study was approved by the local ethics committee (Catholic University Ethics Committee, Rome). The ethical principles of the declaration of Helsinki were strictly followed.

**Histopathology**

All histological samples were reviewed by a board-certified neuropathologist (LL) and all tumors were classified as glioblastoma (WHO IV). Multiple levels of each paraffin block of samples used for research purposes (ET, BAT and CTRL) were thoroughly examined. No tumor cells were seen in BAT samples used in this study.

**Gene Expression Analysis**

For global gene expression analysis we used 4 CTRL samples, 5 ET samples (ET1, ET2, ET3, ET4, ET5), and 7 BAT samples (BAT1, BAT3, BAT5; in two patient samples were taken from two different peritumoral areas: BAT2, BAT2R; BAT4, BAT4R). Total RNA was extracted using Triazol Reagent followed by clean-up and DNase digestion on an RNAeasy spin column. RNA was quantified by UV spectrophotometer and quality was assessed on agarose gel. RNA was processed for use on the Affymetrix Human Expression HG-U133A arrays (Affymetrix, Santa Clara, CA) according to manufacturer’s instructions. Briefly, 2.5 μg of each RNA was converted into double-strand cDNA using a T7-(dT)24 primer. cDNA was used as template to generate biotinylated cRNA during an in vitro transcription step. Labeled cRNA was purified, chemically fragmented and 15 μg were hybridized on the array for 16 h at 45°C in a rotisserie oven set at 60 rpm. The arrays were then stained in the Affymetrix Fluidic Station and scanned twice using the Agilent Gene Array scanner 2500.

The expression data were generated by Affymetrix microarray suite 5.0 software and loaded into GeneSpring Expression Analysis version 7.3 software. Raw intensities from each chip were normalized using the GC-RMA method completed by an additional normalization to the median for each gene. Data were filtered to eliminate genes displaying an averaged intensity inferior to the global array background.

Datasets were then assigned to the three experimental groups: CTRL, ET, and BAT, and the averaged log2 intensities of biological replicates were used for further analysis. Homogeneity of sample groups was verified by analysis of the principal component (PCA). Pre-filtered data were submitted to statistical analysis (t-test; P<0.05 with FDR correction) to identify genes differentially expressed between ET and BAT samples, and between BAT and CTRL. Genes with a fold change of gene expression .2 between CTRL and BAT samples were subjected to hierarchical clustering using Pearson correlation coefficient. Results have been deposited in NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/projects/geo/ accession number: GSE13276).

**Quantitative Real-time PCR**

About 1.5 μg of total RNA were reverse-transcribed by SuperScript III, using random hexamer primers (Invitrogen, Carlsbad, CA, USA). Quantitative real-time PCR (qPCR) was carried out with LightCycler technology (Roche Molecular Biochemicals, Indianapolis, IN, USA). Oligonucleotide primers (Table 1) were designed using Primer3 software (http://frodo.wi.mit.edu/). Cycling conditions: 95°C for 10 minutes, 40 cycles of 95°C for 10 seconds, 58°C for 7 seconds, 72°C for 8 seconds, followed by a melt curve analysis immediately begun to rule out synthesis of unspecific products. Crossing points (Cp) of

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**Figure 1. Tissue sampling.** (A) Tissue specimen opened in a book-wise fashion before sampling; white asterisk: tumor; black asterisk: BAT. Sampling-grid: each tissue specimen was generally divided into eight parts, and BAT samples for molecular analyses were contiguous to those used for histology; in this way, a higher probability of homogeneity between samples used for histology and gene expression analysis, quantitative real-time PCR, western blot analysis and array-CGH was obtained. (B) H&E staining of BAT with absence of morphologically neoplastic cells. (C) GFAP staining, showing reactive astrocytes, with stellar morphology. (D) Ki67/MIB-1 was always <1%. (E) Gene microarray analysis.

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real-time-PCR curves were determined by the Light cycler software using the second derivative maximum method. For each target, two independent amplifications were performed and the mean value was used for further analysis. The $2^{-\Delta\Delta Ct}$ method was applied to calculate fold changes in the expression levels of the genes. Statistical analysis was performed using t-test.

Western-blot Analysis
The presence of KLRC1 (killer cell lectin-like receptor sub family C, member1) in 3 independent specimens of BAT and 3 CTRL samples was evaluated by western-blot analysis. Forty μg of proteins were resolved by 12% SDS-PAGE and transferred to PVDF membrane. Non specific binding was blocked with 5% nonfat dry milk for 1 h. The blots were probed with a 1:500 dilution of mouse anti-human KLRC1 antibody (Abnova Corporation,) or a 1:10.000 dilution of mouse anti-human β-actin, overnight at 4°C. Biotinylated anti-mouse IgG (dilution 1:1000; Vector Laboratories, Burlingame, CA, USA) was used as secondary antibody for 1 h at RT. After washes in PBS containing 0.1% Tween-20, membranes were incubated for 30 min at RT with Vectastain Elite ABC reagent (Vector Laboratories), and antibody reactivity was visualized by incubation with diaminobenzidine.

Array-CGH
DNA from 3 frozen ET samples (ET1, ET2 and ET3) and 3 matched BAT samples (BAT1, BAT2R and BAT3) was extracted using QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). The rest of frozen tissue of BAT1, BAT2R and BAT3 samples was then evaluated in frozen sections and no tumor cells were identified.

Oligonucleotide aCGH was performed using the Agilent Human Genome CGH microarray 4×44K, (Agilent Technologies Santa Clara, CA, USA), with an average resolution of 75 kb, following the manufacturer’s instructions. Each sample was paired with a sex-matched commercial control DNA (Promega, Madison, WI, USA), and hybridized twice in a dye-swap experiment, in order to minimize false positive calls. The arrays were analyzed using GenePix 4000B scanner (Axon, Union City, CA, USA) and Feature Extraction V.9.5.1 software. A graphical overview of the results was obtained using CGH Analytics V.3.5.14 software. Combined dye-swapped experiments were analyzed with ADM-2 algorithm at 3 different thresholds: 4, 5 and 6, with Centralization and Fuzzy Zero corrections turned on, with the purpose of detecting low grade mosaicisms. The percentage of abnormal cells was inferred using the formula proposed by Valli et al. [12].

Immunohistology
Both immunohistochemical and immunofluorescence analyses were performed in paraffin-embedded tissue sections (5 μm thick) from ET and BAT. All samples were deparaffinized and rehydrated. Immunohistochemical and immunofluorescence analyses were performed considering the results of the gene expression analyses. In particular, the expression of some proteins whose genes were over-expressed in the BAT compared with CTRL was analyzed. Moreover, immunofluorescence analysis for CD133 protein was performed.

For immunohistochemistry, after the endogen peroxidase blocking, sections were incubated with monoclonal anti-human GFAP (1:100; clone 273807; R&D SYSTEMS, Minneapolis, MN, USA), anti-human Ki-67 (1:100; clone MIB-1; Dako, Table 1. Primer sequences.

| Gene   | Primer forward (5'–3')                  | Primer reverse (5'–3')                |
|--------|-----------------------------------------|---------------------------------------|
| TAZ    | CAGCAATGTGGGAGTGGGAGTGG                 | TCATTGGAAGGAGCGAGATCGA                |
| KLRC1  | CATCCTCATGATGATGATG                    | GATCCACCTGGGCTGATTT                  |
| EGFR   | TGCTGGATAGACGCGAGA                     | GGCACGTTAGAAGTGGAGT                   |
| IGFBP5 | CAGGGTTGACGCTCTTTGAA                   | GAGGAAGGGCCGAGACGATCT                |
| SRP1   | TGGAGGCGAGGTGAGAGTATG                  | CCGGCTGACACCTCTGCCGTC                 |
| USH1C  | GAAGAAGACTGGGCGTCTCAAA                 | AAGGTGTGCCTCCCGTCTC                   |
| ID3    | GGAGCATTTTGGCATGCTC                   | CAGGAAGGGATTTGGTGAAG                |
| 18S ribosomal | Qiagen Hs_RRN18S_1_SG QuantiTect_Primers Assay (QT00199367) | |

Figure 2. PCA 3D view for gene expression profiles of samples of the three experimental conditions (red: CTRL; yellow: ET; blue: BAT). Every dot represents a sample. PCA was based on log2 ratios and the expression profiles were performed across the 14,500 genes of the human HG-U133A array. The first 3 principal components are plotted. PCA representation shows samples segregation according to their tissue origin.
Table 2. Selection of the genes significantly different between ET and BAT with a 10-fold difference in expression levels.

| Genebank   | Description                                         | Symbol   | FC  | Corrected p-value |
|------------|------------------------------------------------------|----------|-----|-------------------|
| NM_006501  | myelin-associated oligodendrocyte basic protein     | MOBP     | −34.4 | 0.006             |
| NM_003027  | SH3-domain GRB2-like 3                              | SH3GL3   | −31.7 | 0.022             |
| NM_002385  | myelin basic protein                                 | MBP      | −29.2 | 0.029             |
| NM_002433  | myelin oligodendrocyte glycoprotein                 | MOG      | −24.2 | 0.018             |
| NM_016533  | ninjurin 2                                            | NINJ2    | −16   | 0.039             |
| NM_008480  | glutamate receptor, metabotropic 3                   | GRM3     | −13.8 | 0.035             |
| NM_003360  | UDP glycosyltransferase 8                            | UGT8     | −12.9 | 0.004             |
| NM_012294  | Rap guanylnucleotide exchange factor 5               | RAPGEF5  | −12   | 0.03              |
| NM_002371  | mal, T-cell differentiation protein                  | MAL      | −30.6 | 0.017             |
| AL524520   | G protein-coupled receptor 49                        | GPR49    | −12.7 | 0.045             |
| T16257     | G protein-coupled receptor 37                        | GPR37    | −12.6 |                  |
| NM_005709  | Usher syndrome 1C                                     | USH1C    | −10   | 0.014             |
| L35594     | autotaxin                                            | ATX      | −22.3 | 0.037             |
| X98405     | myelin associated glycoprotein                       | MAG      | −18.7 | 0.018             |
| U88870     | peanut-like 2                                         | PNUTL2   | −14.7 | 0.042             |
| NM_016950  | sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 3 | SPOCK3   | −11   | 0.006             |
| NM_003628  | plakophilin 4                                         | PKP4     | −10.8 | 0.017             |
| NM_001063  | transferrin                                          | TF       | −15   | 0.004             |
| NM_018478  | dysbindin                                            | DBNDD2   | −12.4 | 0.026             |
| NM_012128  | chloride channel, calcium activated, family member 4 | CLC4A    | −11.8 | 0.004             |
| NM_007168  | ATP-binding cassette, sub-family A member 8         | ABCA8    | −11.3 | 0.007             |
| BC000585   | solute carrier organic anion transporter family, member 3A | SLC3A1  | −10   | 0.022             |
| NM_002774  | kallikrein 6                                         | KLK6     | −32   | 0.004             |
| NM_002570  | paired basic amino acid cleaving system 4            | PACE4    | −28.5 | 0.043             |
| NM_000049  | aspartoacylase                                        | ASPA     | −24.2 | 0.032             |
| NM_004476  | folate hydrolase 1                                   | FOLH1    | −18.5 | 0.031             |
| NM_004616  | transmembrane 4 superfamily member 3                 | TM4SF3   | −10.8 | 0.005             |
| NM_014682  | suppression of tumorigenicity 18                     | ST18     | −30   | 0.028             |
| NM_014717  | zinc finger protein 536                              | ZNF536   | −16.3 | 0.03              |
| NM_013279  | chromosome 11 open reading frame 9                   | C11orf9  | −10.7 | 0.017             |
| AU157109   | KIAA1598 protein                                      | KIAA1598 | −14.8 | 0.048             |
| AW242297   | microtubule-associated protein 7                     | MAP7     | −13.2 | 0.037             |
| AA191573   | synaptotagin 2                                        | SYNJ2    | −35.4 | 0.022             |
| AB032987   | PAIP2B HGNCC binding protein interacting protein 2B  | PAIP2B   | −27   | 0.047             |
| AB007880   | KIAA0420 gene product                                | KIAA0420 | −22.5 | 0.039             |
| AB080302   | LIM domain binding 3                                 | LDB3     | −20.4 | 0.028             |
| U56725     | Human heat shock protein mRNA.                       | HSPA2    | −20   | 0.038             |
| BC003169   | calpain 3, (p94)                                      | CAPN3    | −18.2 | 0.027             |
| NM_009954  | prostaglandin D2 synthase 21 kDa (brain)             | PTGDS    | −17.6 | 0.026             |
| NM_024306  | fatty acid 2-hydroxylase                             | FABH2    | −13.5 | 0.038             |
Glostrup, Denmark) antibodies or with a polyclonal anti-human TAZ antibody (1:60; LifeSpan Biosciences, Seattle, WA, USA) overnight at 4°C. Subsequently, slides were incubated with a HRP/Fab polymer conjugate (SuperPicTure Polymer Detection Kit, Invitrogen, Camarillo, CA, USA). The immunostaining for Epidermal Growth Factor Receptor (EGFR) and CD99 was performed using the monoclonal antibodies anti-human EGFR (1:100; Clone E30; Dako) and CD99 (1:100; clone 12E7; Dako) on an autostainer (Dako Autostainer Plus Link, Dako). The antigen retrieval was performed using pronase digestion (ProTaqs H P Pronase Digest, Germany) and En Vision TM Flex Target Retrieval Solution High pH (Dako) for EGFR and CD99, respectively.

Immunopositive cells were visualized by brown DAB (Vector Laboratories, Inc., Burlingame, CA, USA) staining. The nuclei were lightly counterstained with Mayer’s hematoxylin. Tonsil sections were used as positive controls for EGFR and CD99 expression.

For CD133 immunofluorescence analysis on ET and BAT, the sections were incubated for 12 h at 4°C with the polyclonal anti-human CD133 (1:50; Spring Bioscience, CA, USA) and were then treated with the secondary antibody (goat anti-rabbit Alexa Fluor 488; 1:200; Invitrogen) for 1 h at room temperature (RT). For double-labeling immunofluorescence analysis, histological sections were incubated for 20 h at 4°C with the anti-GFAP and TAZ antibodies as described above. The next day, the slides were incubated with a mixture of the following secondary antibodies: goat anti-rabbit Alexa Fluor 488 (1:250; Invitrogen) and red fluorescent cyanine donkey anti-mouse Cy3 (1:200; Jackson Immunoresearch Laboratories) for 2 h at RT. The sections were coverslipped with Vectashield Mounting Medium with DAPI (Vector Laboratories) and examined with a confocal laser scanning system (TCS-SP2, Leica Microsystems, GmbH, Wetzlar, Germany) equipped with an Ar/ArKr laser and a HeNe laser for 488 nm and 543 nm excitation, respectively. For each analyzed field, Z-stack series of 4–5 μm-thick were acquired as images (1024×1024 pixels), recorded at intervals.

Table 2. Cont.

| Genebank Description          | Symbol | FC  | Corrected p-value |
|-------------------------------|--------|-----|-------------------|
| phospholysine phosphohistidine inorganic pyrophosphate phosphatase | LHPPP  | −13 | 0.041             |
| synaptotagin 2                | SYNI2  | −12 | 0.027             |
| ectropic viral integration site 2A | EVI2A  | −11.7 | 0.039           |
| progestin and adipQ receptor family member VI | PAQRI6 | −11 | 0.002           |
| breast carcinoma amplified sequence 1 | BCAS1  | −10.6 | 0.015           |
| pentaxin-related gene, rapidly induced by IL-1 beta | PTX3  | 18.2 | 0.028           |
| complement component 1,q subcomponent, receptor 1 | C1QR1  | 16.2 | 0.039           |
| collagen, type I, alpha 2    | COL1A2 | 26.8 | 0.014           |
| collagen, type IV, alpha 2   | COL4A2 | 13.9 | 0.017           |
| collagen, type I, alpha 1    | COL1A1 | 13.0 | 0.018           |
| collagen, type IV, alpha 1   | COL4A1 | 11.8 | 0.014           |
| matrix GlA protein           | MGP    | 10.4 | 0.002           |
| fibronectin 1                | FN1    | 10.2 | 0.033           |
| collagen, type III, alpha 1  | COL3A1 | 10.0 | 0.008           |
| vascular endothelial growth factor | VEGF | 16.0 | 0.032           |
| angiopoietin 2               | ANGPT2 | 10.4 | 0.004           |
| CDC28 protein kinase regulatory subunit 2 | CKS2  | 12.6 | 0.017           |
| insulin-like growth factor binding protein 2, 36 kDa | IGFBI | 35.8 | 0.017           |
| tissue inhibitor of metalloproteinase 1 | TIMP1 | 16.6 | 0.037           |
| growth associated protein 43 | GAP43  | 12.9 | 0.039           |
| transforming growth factor, beta-induced, 68 kDa | TGFBI | 26.6 | 0.026           |
| ribonucleotide reductase M2 polypeptide | RRM2  | 21.1 | 0.022           |
| KIAA0101 gene product        | KIAA0101 | 13.3 | 0.046           |
| ADP-riboseylation factor-like 7 | ARL7  | 10.7 | 0.006           |
| actinin, alpha 1             | ACTN1  | 10.2 | 0.015           |

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For double-labeling immunofluorescence analysis, histological sections were incubated for 20 h at 4°C with the anti-GFAP and TAZ antibodies as described above. The next day, the slides were incubated with a mixture of the following secondary antibodies: goat anti-rabbit Alexa Fluor 488 (1:250; Invitrogen) and red fluorescent cyanine donkey anti-mouse Cy3 (1:200; Jackson Immunoresearch Laboratories) for 2 h at RT. The sections were coverslipped with Vectashield Mounting Medium with DAPI (Vector Laboratories) and examined with a confocal laser scanning system (TCS-SP2, Leica Microsystems, GmbH, Wetzlar, Germany) equipped with an Ar/ArKr laser and a HeNe laser for 488 nm and 543 nm excitation, respectively. For each analyzed field, Z-stack series of 4–5 μm-thick were acquired as images (1024×1024 pixels), recorded at intervals...
of 0.20 μm, and then projection images were created and processed using the Leica software. MDA-MB-231, a breast cancer cell line expressing moderate TAZ levels, was used as positive control for TAZ expression [13]. In each experiment, negative controls without the primary antibody were included to check for nonspecific staining.

Results

Histopathology

The CTRL samples displayed a variable degree of gliosis due to bleeding of the cavernomas and subsequent edema, as it was indicated by the presence of GFAP-reactive astrocytes, with a characteristic dendritic morphology and abundant eosinophilic cytoplasm and with large eccentric nuclei (data not shown). The number of Ki67/MIB1 positive cells was lower than 1% (data not shown).

In the ET samples, the number of Ki67/MIB1 positive cells varied between 10% and 60% (data not shown).

Microarray Analysis

Genome-wide expression profiles of 5 ET samples, 7 BAT samples from 5 patients, and 4 CTRL white matter biopsies were analysed. The representation of the samples in a three-dimensional (3D) space clearly distinguished 3 groups corresponding to the 3
| Genebank         | Description                              | Symbol | FC  | Corrected p-value |
|------------------|------------------------------------------|--------|-----|-------------------|
| **Genes up-regulated in BAT compared to CTRL** |                           |        |     |                   |
| **Cell growth and proliferation** |                           |        |     |                   |
| AW157070        | epidermal growth factor receptor         | EGFR   | 8.0 | 0.0389            |
| AW007532        | Homo sapiens cDNA clone IMAGE:2500861    | IGFBP5 | 2.8 | 0.0253            |
| NM_001321       | cysteine and glycin-rich protein 2       | CSP2   | 2.4 | 0.0314            |
| NM_001392       | Homo sapiens dystrobrevin, alpha         | DTNA   | 2.4 | 0.0295            |
| NM_002167       | inhibitor of DNA binding 3               | ID3    | 3.3 | 0.0468            |
| AI313324        | histone H2A.1                            | HIST2H2AA | 2.6 | 0.0468            |
| **Cell adhesion/motility** |                           |        |     |                   |
| NM_001078       | vascular cell adhesion molecule 1        | VCAM1  | 3.4 | 0.0159            |
| U82164          | CD99 antigen                             | CD99   | 2.7 | 0.0218            |
| NM_005709       | Usher syndrome 1C                        | USH1C  | 2.6 | 0.0192            |
| NM_021077       | neuromedin B                             | NMB    | 2.3 | 0.0468            |
| BF674349        | transcriptional co-activator with PDZ-binding motif | TAZ   | 2.7 | 0.0468            |
| AU157932        | palladin                                 | PALLD  | 2.3 | 0.0256            |
| **Apoptosis**   |                           |        |     |                   |
| NM_005460       | synuclein, alpha interacting protein     | SNCAIP | 2.8 | 0.05              |
| **Unknown function** |                           |        |     |                   |
| AU154455        | Homo sapiens cDNA clone NT2RP4001145     | T1A-2  | 2.4 | 0.0314            |
| NM_022074       | FLJ22794 protein                         | FLJ22794 | 2.1 | 0.0192            |
| **Genes down-regulated in BAT compared to CTRL** |                           |        |     |                   |
| **Angiogenesis** |                           |        |     |                   |
| NM_001704       | brain-specific angiogenesis inhibitor 3  | BAI3   | −2.8| 0.0468            |
| **Transcription** |                           |        |     |                   |
| AF208967        | paternally expressed 3                   | PEG3   | −5.6| 0.0159            |
| AI810712        | hepatic leukemia factor                  | HLF    | −5.5| 0.0496            |
| NM_004538       | nucleosome assembly protein 1-like 3     | NAP1L3 | −3.3| 0.0496            |
| AL136629        | TSPY1-like                               | TSPYL1 | −3.2| 0.0468            |
| AV721430        | transcription factor 7-like 2            | TCF7L2 | −2.3| 0.0496            |
| AL096375        | KIAA1750 protein                         | TSPYL5 | −2.3| 0.0168            |
| NM_012231       | PR domain containing 2, with ZNF domain  | PRDM2  | −2.5| 0.0496            |
| AA488899        | protein associated with Myc               | MYCBP2 | −2.0| 0.00518           |
| BG402105        | RB1-inducible coiled-coil 1              | RB1CC1 | −2.5| 0.0192            |
| Z98884          | calmodulin binding transcription activator 1 | CAMTA1 | −2.6| 0.0468            |
| AB020663        | DmTx-like 2                              | DMXL2  | −2.0| 0.0496            |
| AL050331        | Human DNA sequence from clone RP3-486I3 on chromosome 6q22.1–22.3 | TSPYL4 | −2.2| 0.0314            |
| **Signal transduction** |                           |        |     |                   |
| NM_002738       | protein kinase C, beta 1                 | PRKCB1 | −11.3| 0.0496           |
| NM_007023       | RAP guanine-nucleotide-exchange factor 4  | RAPGEF4 | −7.0| 0.0496           |
| NM_000807       | gamma-aminobutyric acid (GABA) A receptor, alpha 2 | GABRA2 | −4.4| 0.0158           |
| NM_015678       | neurobeachin                             | NBEA   | −3.5| 0.0496           |
| AB020717        | synaptoplakin 1                          | SYNJ1  | −2.9| 0.0468           |
| BC000498        | aspartate aminotransferase 1             | GOT1   | −2.7| 0.0496           |
| AB007896        | putative L-type neutral amino acid transporter | KIAA0436 | −2.6| 0.0159           |
| NM_012093       | adenylyl kinase 5                        | AK5    | −2.5| 0.0468           |
| L39833          | potassium voltage-gated channel, shaker-related subfamily, beta member 1 | KCNAB1 | −2.4| 0.0496           |
| NM_014710       | G protein-coupled receptor associated sorting protein | GASP | −2.2| 0.0496           |
tissues (Figure 2). The CTRL and BAT samples were pooled in 2
distinct groups.

Differential Gene Expression between ET and BAT

Molecular analysis of ET and BAT samples showed significant
changes in the expression of 1,323 genes. Genes with at least 10-
fold difference in the expression level were further analyzed
(Table 2). Twenty genes were over-expressed in ET when
compared to BAT, while 45 genes were down-regulated. The
expression of the angiogenesis-related genes
\textbf{VEGF} and \textbf{ANGPT2} was increased in ET. Genes associated with cell growth (\textbf{IGFBP2},
\textbf{GAP43}), along with the cell cycle activator
\textbf{CKS2}, were over-expressed. Most of the highly up-regulated genes encoded proteins
associated with the extracellular matrix formation, including
\textbf{COL4A1}, \textbf{COL4A2}, \textbf{COL1A1}, \textbf{COL3A1}, \textbf{COL1A2}. The 45 genes
showing significantly decreased expression in ET were more
heterogeneous with respect to the functions of their gene products.
The majority of these genes were involved in the development of the
nervous system (\textbf{MOG}, \textbf{RAPGEF2}, \textbf{GRM3}, \textbf{SH3GL3}, \textbf{NINJ2},
\textbf{UGT8}, \textbf{MOBP}, \textbf{MBP}).

Differential Gene Expression between BAT and CTRL

Statistical analysis associated with a threshold approach (cut-off:
2) resulted in 37 genes showing significantly different expression in
BAT and CTRL. This dataset was submitted to hierarchical
clustering to determine the gene expression pattern in all tissue
 types, including also ET specimens (Figure 3).

Molecular profiling showed 15 genes over-expressed and 42
genes down-regulated in BAT (Table 3). Genes belonging to 2
main relevant biological processes were particularly deregulated in
BAT. In fact, genes associated with growth and proliferation
(\textbf{CSRP2}, \textbf{TAZ}, \textbf{ID3}, \textbf{DTNA}) and cell motility/adhesion
(\textbf{HIST2H2AA}, \textbf{EGFR}, \textbf{IGFBP5}, \textbf{VCAM1}, \textbf{CD99}) were up-regulated
while genes involved in neurogenesis (\textbf{SYNJ1}, \textbf{NBEA}, \textbf{SERPINI1},
\textbf{CNTNAP2}, \textbf{RELN}) were largely down-regulated in BAT. Several
tumour suppressor genes (\textbf{BAI3}, \textbf{PEG3}, \textbf{PRDM2}, \textbf{RB1CC1}) along
with the natural killer receptor \textbf{KLRC1} were also down regulated in
BAT.

Validation of Gene Expression by RT-PCR

A subset of 7 genes was selected for further analysis by qPCR
(Figure 4a). The $2^{-\Delta\Delta C_t}$ method was used to calculate fold
changes in the expression levels of the selected genes in the
comparison BAT vs CTRL samples. The qPCR results showed that
the expression levels of \textbf{ID5}, \textbf{TAZ}, \textbf{EGFR}, \textbf{IGFBP5}, \textbf{USH1C}
were increased in BAT samples, while the expression of \textbf{SERPINI1}
and \textbf{KLRC1} was decreased. These results supported the validity of
microarray results. \textbf{KLRC1} was selected to assess differences in
protein levels between BAT and CTRL samples by the means of
western blot analysis (Figure 4b). \textbf{KLRC1} was detected in all

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{Genebank} & \textbf{Description} & \textbf{Symbol} & \textbf{FC} & \textbf{Corrected p-value} \\
\hline
NM_014247 & Rap guanine nucleotide exchange factor 2 & RAPGEF2 & $-2.1$ & 0.0131 \\
AF052117 & chloride channel 4 & CLCN4 & $-2.1$ & 0.0468 \\
AB002390 & lysosomal aspartate-like 1 & LYSAL1 & $-2.0$ & 0.0302 \\
\hline
\textbf{Extracellular matrix remodelling} & & & & \\
NM_000202 & iduronate 2-sulfatase & IDS & $-3.0$ & 0.0496 \\
AU144167 & collagen, type III, alpha 1 & COL3A1 & $-2.7$ & 0.0468 \\
\hline
\textbf{Growth} & & & & \\
NM_020988 & guanine nucleotide binding protein alpha activating activity polypeptide O & GNAO1 & $-2.1$ & 0.0192 \\
NM_00345 & synuclein, alpha & SNCA & $-2.1$ & 0.0496 \\
NM_005025 & serine protease inhibitor & SERPIN1 & $-2.0$ & 0.0192 \\
\hline
\textbf{Adhesion} & & & & \\
AW190070 & ATPase, Ca++ transporting, cardiac muscle, slow twitch 2 & ATP2A2 & $-2.5$ & 0.0496 \\
AU144598 & contactin associated protein-like 2 & CNTNAP2 & $-2.4$ & 0.0453 \\
NM_005045 & reelin & RELN & $-2.2$ & 0.035 \\
\hline
\textbf{Cytoskeleton} & & & & \\
AB011133 & microtubule associated serine/threonine kinase 3 & MAST3 & $-2.2$ & 0.0158 \\
\hline
\textbf{Defense} & & & & \\
NM_002260 & killer cell lectin-like receptor subfamily C, member 1 & KLRC1 & $-5.0$ & 0.0125 \\
\hline
\textbf{Ubiquitin cycle} & & & & \\
AV726900 & Ring finger protein 20 & RNF20 & $-2.5$ & 0.00518 \\
AL031178 & F-box protein 9 & FBXO9 & $-2.2$ & 0.0496 \\
NM_018422 & pleckstrin and Sec7 domain containing 3 & PSD3 & $-2.5$ & 0.0314 \\
\hline
\textbf{Unknown} & & & & \\
AL050136 & Homo sapiens mRNA; cDNA DFFZpS66L141 & & $-2.2$ & 0.0352 \\
NM_014951 & Zinc finger protein 365 & ZNF365 & $-2.4$ & 0.0192 \\
AF063591 & CD200 molecule & CD200 & $-2.2$ & 0.0159 \\
\hline
\end{tabular}
\caption{Cont.}
\end{table}
CTRL specimens, while a very weak signal was detectable in the BAT samples.

Array CGH
A large number of mosaic genomic lesions were detected by aCGH in tumor samples. Some of these alterations, such as del (1p36), del (2p21), +7, del (6q27q29)/2, EGFR (7p11.2), MDM2 (12q15) and CDK4 (12q14.3) amplification, 2 amplification of 15q24.1, del (17p13)/2, −17, −19, −22 were known to be associated with glioblastomas [14,15].

Several tumor-associated lesions were also present in the BAT. Shared anomalies with ET were del(1p36), del(2p21), MDM2 and CDK4 amplification, amplification of 15q24.1, del (17p13)/−17, −19, −22 were known to be associated with glioblastomas [14,15].

Several tumor-associated lesions were also present in the BAT. Shared anomalies with ET were del(1p36), del(2p21), MDM2 and CDK4 amplification, amplification of 15q24.1, −19 and −22. The genetic changes of +7, del(6q27q29), EGFR amplification, −10, del (17p13) were limited to the ET. The summary of these results is shown in Table 4.

Immunohistology
TAZ expression was found in the majority of GBM cells, predominantly in the cell nuclei (Figure 5C). Double-labelled ET samples revealed GFAP and TAZ co-expression when analyzed by confocal microscopy (Figure 6A–C). The majority of neoplastic cells expressed TAZ in the nucleus, and only few cells showed a cytoplasmatic staining (Figure 6B, C). As expected, GFAP was highly expressed in the cytoplasm of tumor cells (Figure 5A; Figure 6A, C). In ET samples, as expected, the majority of tumor cells exhibited strong specific EGFR immunopositivity (Figure 7A).

The reactivity was strong on the cell membrane and less intense in the cytoplasm of the tumor cells. The same expression pattern was found for CD99 in the GBM cells (Figure 7D).

In BAT, GFAP immunostaining was displayed in both apparently normal and reactive astrocytes (Figure 1C; Figure 5B). The frequency of Ki67/MIB1 positive cells was always lower than 1% (Figure 1D). TAZ immunopositive nuclei were extremely rare (Figure 5D). Confocal microscopy of BAT samples confirmed that expression of TAZ was rarely seen in peritumoral tissue samples (Figure 6E, F), while apparently normal and reactive astrocytes were positive for GFAP staining (Figure 6D, F). In BAT, the EGFR immunopositivity was observed in reactive astrocytes (Figure 7B) as well as for CD99 that was also expressed in some normal cells (Figure 7E). The mean percentage of EGFR- and CD99-positive cells was higher in BAT (10.1 ± 2.6 for EGFR and 11.5 ± 2.8 for CD99) with respect to CTRL (3.2 ± 0.1 and 2.7 ± 0.2 for EGFR and CD199, respectively; Figure 7C, F).

In ET samples, CD133 cytoplasmic immunopositivity, visualized by immunofluorescence microscopy, was found in a moderate number of tumor cells (Figure 8 A). In BAT, the same pattern of reactivity was observed in a low percentage (1.9 ± 0.3) of reactive astrocytes and apparently normal cells (Figure 8 B). A very low value (0.06 ± 0.1) of CD133-positive cells was displayed in CTRL samples (Figure 8C).
**Table 4.** Consistent anomalies observed in ET and BAT by a-CGH.

| Consistent anomalies | ET1 | BAT1 | ET2 | BAT2 | ET3 | BAT3 |
|----------------------|-----|------|-----|------|-----|------|
| del (1p36)           | +   | [27645717–29259696] (40%) | +   | [27645717–29259696] (45%) | +   | [1009416–46806902] (40%) | +   | [1009416–46806902] (45%) |
| del (2p21)           | –   | –    | –   | –    | –   | [47450573–47510722] (64%) | –   | [46985724–47510722] (70%) |
| +7                   | –   | –    | +   | –    | –   | –    | –   | – |
| del (6q27q29)/–6     | +   | Complete monosomy (34%) | –   | –    | –   | [163542007–166844004] (52%) | –   | – |
| amp EGFR (7p11.2)    | –   | –    | +   | [54571903–55349837] | –   | –    | –   | – |
| − 10                 | −   | –    | +   | (60%) | –   | + (80%) | –   | – |
| amp CDK4 (12q14.3)   | +   | [64510897–64589846] | +   | [64510897–64589846] | –   | –    | –   | – |
| amp MDM2 (12q15)     | +   | [67369376–68348402] | +   | [67369376–68348402] | –   | –    | –   | – |
| amp 15q24.1          | +   | [70483070–71401705] | +   | [70764425–71255997] | –   | –    | –   | – |
| del (17p13)(p53)/–17 | +   | [505704–40281123] (17%) | –   | Complete monosomy (20%) | –   | Complete monosomy (40%) | –   | – |
| − 19                 | +   | (30%) | –    | [19%(19%) | –   | –    | –   | [15%(15) |
| − 22                 | +   | (38%) | –    | (23%) | –   | + (23%) | –   | + (20%) |

*Further amplification in ET, with respect to BAT.

In bold: anomalies limited to ETs.

In square bracket: genomic positions (according to NCBI 36 build) of the observed anomalies.

In round brackets: mosaicism degree of the observed anomaly. The percentage of abnormal cells was inferred using the formula proposed by Valli et al. That formula cannot be applied to the amplified segments (EGFR, CDK4, MDM2 and 15q24.1) since their ploidy status is unknown.

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Figure 5. Immunohistochemical staining for GFAP and TAZ in ET and BAT. The majority of GBM cells showed intense diffuse cytoplasmic staining for GFAP (A). In the BAT, only apparently normal and reactive astrocytes (black arrow) expressed GFAP protein (B). TAZ immunoreactivity, mainly nuclear, was uniformly expressed in the cells of the ET (C). In the BAT, TAZ positive cells were observed very infrequently (D). Original magnification, ×630 (A–D). Hematoxylin counterstain.
doi:10.1371/journal.pone.0057145.g005
Figure 6. Confocal microscopy images of GFAP (red) and TAZ (green) expression in ET and BAT. In the ET samples, GFAP was highly expressed in the cytoplasm of neoplastic cells (A, C). In the BAT, it was present in the body and cytoplasmic extensions of astrocytes (D, F). In the ET, TAZ was expressed predominantly in the nucleus (white arrowheads) and few cells also showed a cytoplasmic localization (yellow arrowhead) (B, C). Rarely, the GBM cells were negative for TAZ (arrow) (B, C). In the BAT, TAZ was almost undetectable. In the photograph shown, no expression of TAZ was observed (E, F). Scale bar: 20 μm. doi:10.1371/journal.pone.0057145.g006

Figure 7. Immunohistochemical staining for EGFR and CD99 in ET, BAT and CTRL. In the ET samples, tumor cells showed intense staining for EGFR, mainly in the cell membrane (A). In the BAT, black arrow points to EGFR immunopositive reactive astrocytes (B). In the ET, CD99-immunoreactivity can be observed at the membrane level and in the cytoplasm (D). In the BAT, CD99 immunopositivity was found in reactive astrocytes (black arrow), in some normal glial cells (black arrowhead) (E). In the CTRL, immunoreactivity for EGFR or CD99 was rarely observed (C, F). Original magnification: x630 (A-B, D-E), x200 (C, F). Hematoxylin counterstain. doi:10.1371/journal.pone.0057145.g007
Discussion

Few information is available about the peritumoral tissue sampled at least one cm from the macroscopic tumor border. By comparing the expression pattern of CTRL and BAT, we separated 57 genes which were differentially expressed in BAT against CTRL. These genes were also highly expressed in GBM suggesting that GBM and morphologically normal appearing BAT share a similar expression profile. In our study, the EGFR expression levels showed the largest difference between CTRL and BAT, being highly expressed in the latter. The EGFR gene is the most frequently amplified proto-oncogene in primary glioblastomas [16]. Another growth factor receptor, NMB, was also overexpressed in BAT. We also found in BAT an up-regulation of IGFBP5, histone HIST2H2AA and transcription regulator ID3. All these elements are involved in proliferation and tumor progression [17,18,19].

In BAT, we also detected an over-expression of genes involved in cell motility, such as palladin, alpha-dystrobrevin (DTNA), CD99 and VCAH-I [17,20,21,22,23,24]. We also found the over-expression of the transcription regulator TA2 in BAT. This molecule controls the expression of genes regulating cell migration and proliferation [13,25]. TA2 association with mesenchymal (MES) gene expression signature of glioblastoma, resulting in poor overall survival and treatment resistance has recently been emphasized [26].

The set of genes showing reduced expression in BAT included several ones previously described for their anti-oncogenic role, controlling intracellular signalling cascade and transcription molecular functions. Among these, the expression of BA13, PEG3, SV4C has already been reported to be absent or significantly decreased in GBM and glioma cells [27,28,29].

Several genes down-regulated in BAT such as PRDM2, TCFT2L2, RB1CC1, ATP2A2 are involved in the pathogenesis of other type of cancers, but not in gliomas [30,31,32,33,34,35]. Interestingly, SEN71, NBEA, SERPINI1, CNTNAP2 and RELN, which are known to be involved in neurogenesis, were down-regulated in BAT [36,37,38,39,40].

KLRC1, an inhibitory receptor for the non-classical MHC class I molecule HLA-E, has been involved in the inhibition of innate anti-glioma immune responses [41]. In our study, we reported the strong down-regulation of KLRC1 in BAT samples, both at transcriptional and protein level. This finding suggests that an inhibition of a proper immune response may exist. A big limitation of this study is that the “normal” white matter control samples did not come from the same patients bearing the glioblastoma, but from different patients operated for non-neoplastic lesions. The ideal would have been that the white matter controls came from the same patient but very far from the tumor (possibly in the other hemisphere). Obviously, this is not possible in vivo, due to ethical problems, and is not feasible post mortem, because at that point, the spread of the disease possibly involves multiple areas of the brain [42].

The differences between CTRL and BAT and the similarity of gene expression of BAT and GBM could be explained by the presence of infiltrative tumor cells that we were not able to detect at histological analysis. On this way, we performed the aCGH, a technique which is able to detect chromosome alterations only if these are present in a high percentage of cells. By comparing individual ET and BAT, we observed that BAT of two patients (BAT1 and BAT3) showed that almost all cells displayed anomalies consistently associated with GBM, but none of the cells of BAT2 displayed chromosomal anomalies known to be associated with GBM, even in the presence of several dysregulated
genes. However, from a clinical point of view, the outcome of the patient whose BAT did not display any evident genomic change was similar to that of the other two patients. Obviously, in view of the small number of patients in the study, no conclusions on the relationship between genomic alterations of the BAT and survival can be drawn.

Overall, data interpretation is not easy. We can exclude that the differences in genes expression between CTRL and BAT reflect reactive changes as we have found the same level of gliosis, as determined by the presence of GFAP positive reactive astrocytes and the poor macrophages infiltration (data not shown). Undoubtedly, peritumor tissue sampled at 1 cm or more from the macroscopic tumor border presents frank gene/chromosome alterations. Some of these alterations were also present at the protein level, as shown by immunohistological analysis.

As a matter of fact, the mean percentage of EGFR- and CD99-positive cells in BAT was higher than 10%, while CD133 cytoplasmic immunopositivity was observed in a very low percentage (less than 2%) of reactive astrocytes and apparently normal cells. As CD133 is a marker of brain cancer stem cells, our data revealed that the amount of possible CD133 positive cancer stem cells was low in the BAT. Nonetheless, considerable experimental evidence for the existence of both CD133 positive and CD133 negative populations as tumor-initiating cells exists [43]. Therefore, we may have missed some putative cancer cells.

Undoubtedly, the array-based gene expression profile could result from a minority of cells, but this condition is in contrast with the array CGH results, showing chromosomal aberrations in the vast majority of cells.

On the other hand, the correlation between gene overexpression and encoded protein levels can also be weak.

All our experiments suggest that several tumor-like alterations are detectable in the BAT. Nevertheless, question remains about the nature of the cells populating the peritumour tissue. Two main hypotheses may support these findings and these hypotheses are not mutually exclusive. Our data could be explained by a dilution of genes expressed from tumor cells infiltrating the BAT samples: in fact the genes overexpressed in BAT against CTRL were also not mutually exclusive. Our data could be explained by a dilution of genes expressed from tumor cells infiltrating the BAT samples: in fact the genes overexpressed in BAT against CTRL were also over-expressed in GBM but with a higher fold change. In fact, histological analysis may have missed the presence of some infiltrative tumor cells in the BAT. These cells could also be histologically undetectable, “dormant” infiltrating cells. [44].

A possible role of cancer stem cells in determining genetic changes in the BAT is difficult to sustain, since we observed few CD133 positive cells in this area, but we may have missed CD133 negative cancer stem cells.

Alternatively, these findings could be supported by a relevant amount of cells that present a gene profile compatible with a precancerous state. The aCGH data seem to be in favour of this hypothesis. Of course, the truth may lie somewhere in between, and the recurrent tumor may arise from both infiltrating tumor cells (including histologically undetectable tumor cells, “dormant” tumor cells and possibly cancer stem cells) and from an interaction and recruitment of apparently normal cells in the peritumor tissue by infiltrating tumor cells. If the latter two hypotheses are true, our observations could reflect a change of the BAT, which may progress to tumor by acquiring genomic alterations characteristic of GBM. Some changes might be induced by the cross-talk between tumor cells and normal cells. A possible mechanism of this phenomenon can be explained through the transporting of microvesicles containing RNA and a number of mRNA transcripts, which may lead to induction of oncogenic processes in peritumoral normal cells [45]. BAT may also bear traces of the tumor microenvironment in the form of microvesicles or exosomes transporting tumor mRNA or even genomic DNA, which could also partially explain our findings.

Whatever the correct explanation of our findings, further studies on the BAT are needed, in order to identify possible targets for future treatments.

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Author Contributions
Conceived and designed the experiments: AM NS BLP DO MZ CA AC. Contributed reagents/materials/analysis tools: AM NS DO MZ BLP GP AC LL. Wrote the paper: PDB AM NS G. Sica GL DO G. Sica GL DO G. Sica GL GP AC LL.

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