Epidermal Growth Factor Receptor Pathway Gene Expressions and Biological Response of Glioblastoma Multiforme Cell Lines to Erlotinib

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Abstract. *Background: Erlotinib, an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, exerts highly variable antiproliferative effects on human glioblastoma multiforme (GBM) cells in vitro and in vivo. As these effects are independent of EGFR baseline expression levels, more complex genetic signatures may form the molecular basis of the erlotinib-sensitive and erlotinib-resistant GBM phenotypes. The aim of the current study was to determine which genes within the EGFR signaling pathway are candidates for mediating the cellular response of human GBM towards erlotinib. Materials and Methods: Complementary (c)RNAs from cell lines selected to represent the sensitive, intermediately responsive and resistant phenotypes, respectively, were hybridized to CodeLink Human Whole Genome Bioarrays. Results: Expression analysis of the prospectively selected 244 genes whose products constitute the EGFR signaling pathway identified five genes the expression of which significantly correlated with phenotype. Functional annotation analysis revealed one (STAT1) and two (FKBP14, RAC1) genes conclusively associated with sensitivity and resistance to erlotinib, respectively. Moreover, two additional genes (PTGER4, MYC) were unexpectedly found to be associated with sensitivity. The gene expressions were confirmed by quantitative polymerase chain reaction. Conclusion: Five genes within the EGFR signaling pathway may modulate GBM response to erlotinib, which further emphasizes the importance of this pathway for the biology of GBM.*

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Alterations of the epidermal growth factor receptor (EGFR) and its pathway are prominent features of glioblastoma multiforme (GBM) (1, 2). The complex nature of these alterations is highlighted by the fact that erlotinib, an EGFR tyrosine kinase inhibitor, exerts highly variable antiproliferative effects on human GBM cell lines independent of their EGFR baseline expression levels *in vitro* and *in vivo* (3). By analyzing a set of erlotinib-sensitive, intermediately responsive and erlotinib-resistant GBM cell lines, the aim of the current study was to determine genes within the EGFR signaling pathway that may modulate the response of human GBM towards erlotinib.

Materials and Methods

Glioblastoma cell lines. From nine established human GBM cell lines previously described (3), four were selected to represent the erlotinib-sensitive (G-599GM), intermediately responsive (G-210GM and G-750GM) and erlotinib-resistant (G-1163GM) phenotypes, respectively. One additional cell line derived from a secondary GBM (H-199GM) was obtained from Dr. C. Herold-Mende (University of Heidelberg, Germany). The proliferative properties, including response to erlotinib, of this cell line were established as previously described (data not shown) (5) and were indicative of the erlotinib-sensitive phenotype. The cells were maintained in Roswell Park Memorial Institute 1640 cell culture medium (BioWhittaker, Walkersville, MD, USA) supplemented with 10% heat-inactivated fetal calf serum and incubated in a humidified 5% carbon dioxide atmosphere at 37˚C. The medium was exchanged twice weekly and the cells were passaged upon reaching subconfluence. At the beginning of the study, all of the cell lines were beyond their 20th passage. The neuropathological diagnoses of GBM were confirmed by immunocytochemical staining for glial fibrillary acidic protein and vimentin.

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Table I. *Genes with statistically significant correlation between their quantitative expression and biological response of GBM cell lines to erlotinib.*

Gene symbol	Gene name	Main gene function(s) related to cancer
*FKBP*14	FK506-binding protein 14 (22 kDa)	(Inhibition of apoptosis [*FKBP* gene family])[#]
MYC	Myelocytomatosis viral oncogene homolog[‡]	Inhibition of apoptosis
*PTGER*4	Prostaglandin E receptor 4	(Up-regulated in colon cancer metastases)[#]
*RAC*1	Ras-related C3 botulinum toxin substrate 1[‡]	Inhibition of apoptosis
*STAT*1	Signal transducer and activator of transcription 1[§]	Apoptosis; inhibition of proliferation

FK506 = tacrolimus; [#]Parentheses indicate uncertainty within the current knowledge base regarding the exact gene function; [‡]Gene with previously documented relevant role in glioma (see Results and Discussion); [§]Phenotype-related distribution of expression only partially confirmed by qRT-PCR (see Results and Discussion).

RNA extraction. The total cellular RNAs were isolated in three biological replicates from the above cell lines using a spin column system (RNeasy Mini Kit, Qiagen, Hilden, Germany), quantified using spectrophotometry, adjusted to equal concentrations and treated with RNase-free DNase I (Promega, Madison, WI, USA).

RNA processing, microarray hybridization and feature extraction. For microarray target preparation and hybridization, 2 μg of the total cellular RNAs were processed for each of the 15 samples. The Codelink Expression Array Reagent Kit (Applied Microarrays, Tempe, AZ, USA) was used for complementary (c)DNA synthesis and subsequent *in vitro* transcription including biotin-16-uridine triphosphate (Roche Applied Science, Penzberg, Germany). The biotinylated cRNAs were recovered using the RNeasy Mini Kit (Qiagen), spectrophotometrically quantified, and 10 μg per sample were fragmented and hybridized to Codelink Human Whole Genome Bioarrays according to the manufacturer's protocol (Applied Microarrays). After incubation at 37˚C for 18 h, the arrays were washed, stained with Streptavidin-Cy5 (Applied Microarrays) and read using an Axon GenePix 4000B scanner as described in the manufacturer's manual (Molecular Devices, Sunnyvale, CA, USA). The scanned image files were analyzed using the Codelink Expression Analysis software 4.0 (Applied Microarrays).

Statistical analysis and gene annotation. Raw microarray intensity values were preprocessed by performing background correction and cyclic loess normalization using the software R, version 2.5.0 (4). If applicable, up to two missing intensity values per gene were imputed using k-nearest neighbour averaging. For each member of a preselected group of 244 genes whose products constitute the EGFR signaling pathway (5), intensity values were compared between the three different phenotypes (sensitive, intermediately responsive and resistant, respectively). Those values were tested for a monotone trend employing the non-parametric, two-sided Jonckheere-Terpstra test. The Jonckheere-Terpstra test was implemented into a multiple test procedure (6) included in the R software package. This procedure uses a bootstrapping approach to estimate the null distribution of the joint test statistics. The resulting gene-specific p-values were adjusted for multiplicity by controlling the family-wise type I error rate at the 5%-level. Genes with an adjusted $p<0.05$ were referred to as statistically significant candidate genes for response to erlotinib. The list obtained by statistical analysis was submitted to the Database for Annotation, Visualization and Integrated Discovery (DAVID) 2007 (http://david.abcc.ncifcrf.gov/), and sublists were created using DAVID's functional annotation tools.

Confirmatory quantitative reverse transcription-polymerase chain reaction (qRT-PCR). For cDNA synthesis, 50 pg of the total cellular RNA were processed for each of the samples according to the manufacturer's recommendations (QuantiTect Reverse Transcription, Qiagen). Aliquots of the obtained cDNA were added to the real-time PCR mix consisting of the respective gene-specific sense and antisense primers (final concentration of 0.5 pmol/μl per primer) and the PCR master mix (QuantiTect SYBR Green PCR, Qiagen). SYBR green I dye was used for real-time PCR on a 7500 Real-Time PCR System (Applied Biosystems, Darmstadt, Germany) with a final sample volume of 20 μl and the following amplification conditions: 2 minutes at 50˚C and 10 minutes at 95˚C followed by 40 cycles each of 15 seconds at 95˚C and 1 minute at 60˚C. The final cycle consisted of 15 seconds at 95˚C, 1 minute at 60˚C, 15 seconds at 95˚C and 15 seconds at 60˚C. To design primer pairs for the five candidate gene targets with a product size ranging from 69-102 bases, the Universal ProbeLibrary Assay Design Center (Roche Applied Science) and *Primer3* (http://primer3.sourceforge.net) were used. The primers (18-25-mers) were custom-synthesized by Sigma-Aldrich (Munich, Germany). The amount of cDNA was calculated by normalizing the Ct values with those of previously chosen housekeeping genes (hypoxanthine-guanine phosporibosyltransferase 1, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta polypeptide and peptidylprolyl isomerase A), expression of which was determined by parallel analysis.

Results and Discussion

Expression analysis of the prospectively selected 244 genes whose products constitute the EGFR signaling pathway identified five genes the expression of which significantly correlated with phenotype (Table I). The expressions of three candidate genes increased with cellular sensitivity towards erlotinib, and the opposite was observed for the remaining two genes (Figure 1). Functional annotation analysis revealed one (*STAT*1) and two (*FKBP*14, *RAC*1) genes conclusively associated with sensitivity and resistance to erlotinib, respectively (Figure 2). The two candidate genes for conferring GBM resistance to erlotinib potentially represent therapeutic targets within the EGFR signaling pathway, interference with which may enhance the efficacy of erlotinib against GBM. Additionally, two genes (*MYC*, *PTGER*4) were unexpectedly found to be associated with sensitivity (Figure 2). Based on the obvious contradiction between proposed

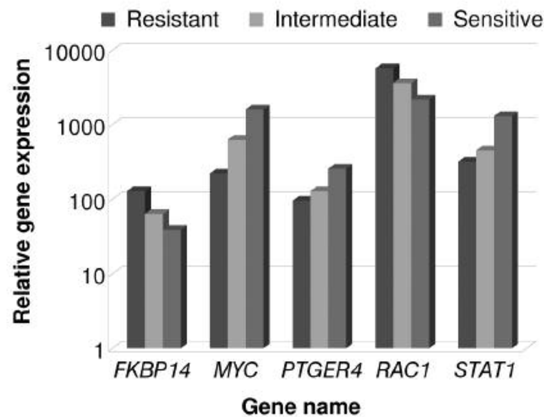

Figure 1. *Gene expressions with significant correlation to biological response of GBM cell lines towards erlotinib as determined by Jonckheere-Terpstra statistics from a group of 244 prospectively selected genes, the products of which constitute the EGFR signaling pathway. Relative gene expressions are mean values of up to two cell lines per phenotype and three biological replicates per cell line. For abbreviations see Table I.*

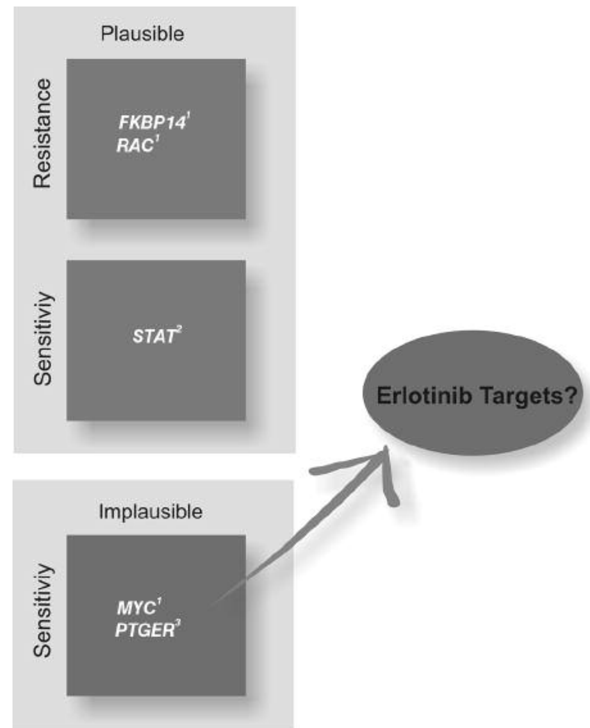

Figure 2. *Candidate genes for GBM response to erlotinib. Sublists were created using DAVID 2007 (see Materials and Methods and Figure 1). Two genes (MYC, PTGER4) were unexpectedly found to be associated with sensitivity (bottom box). For abbreviations see Table I.*

gene functions (Table I) and their expression pattern among the different cellular phenotypes (Figure 1), the products of these genes qualify as putative co-targets of erlotinib.

The virtual localizations of the examined genes within the statistical distribution of correlation between gene expression and categories of cellular response to erlotinib are shown in Figure 3. Two genes (*MYC*, *RAC*1) play established roles in the pathogenesis, maintenance and/or progression of malignant glioma. MYC exerts a well-known critical function in glioma cell proliferation (7, 8) whereas RAC1 is a key contributor to glioma cell survival (9). In contrast to most other members of the STAT family, STAT1 assumes predominantly growth-inhibitory and proapoptotic activity by activating caspase genes, direct interaction with p53 and negative regulation of mouse double minute 2 (MDM2) (10, 11). While the functions of *PTGER*4 and *FKBP*14 have not been as extensively characterized so far, the available data do indicate proneoplastic roles for both genes. For example, *PTGER*4 has been found to be up-regulated in colon cancer carcinomatoses (12). FKBPs, on the other hand, are encoded by a multigene family and generally cause inhibition of apoptosis. In accordance with this notion, a distinct member of this family, *FKBP*12, is overexpressed in childhood astrocytomas (13). To the best of our knowledge, this report is the first to suggest a role of *FKBP*14 in glioblastoma biology.

Gene expressions were confirmed by qRT-PCR (data not shown). Confirmation was unequivocal, with the exception of *STAT*1. For this gene, the quantitative mRNA expression of the intermediately responsive group of cell lines expectedly was 8.61-fold below that of the erlotinib-sensitive phenotype, but also 1.36-fold below that of the erlotinib-resistant cell lines. However, because the difference between *STAT*1 mRNA expressions of the erlotinib-resistant and intermediately responsive cell lines was small compared to *STAT*1 mRNA expression of the erlotinib-sensitive phenotype (*i.e.*, ratio of 0.04), *STAT*1 was retained within the candidate gene list.

By using a unique human GBM cell culture model representing three different compound-related phenotypes, candidate genes within the EGFR signaling pathway for modulating cellular response to erlotinib were identified. Thus, alterations of downstream EGFR signaling pathway elements may play an important role in this regard. Based on the demonstrated statistical association between gene expressions and phenotype, confirmation of these relationships, *e.g.* by transfection experiments, is worthwhile. This line of research may facilitate the rational development of novel targeted compounds with erlotinib-synergistic actions.

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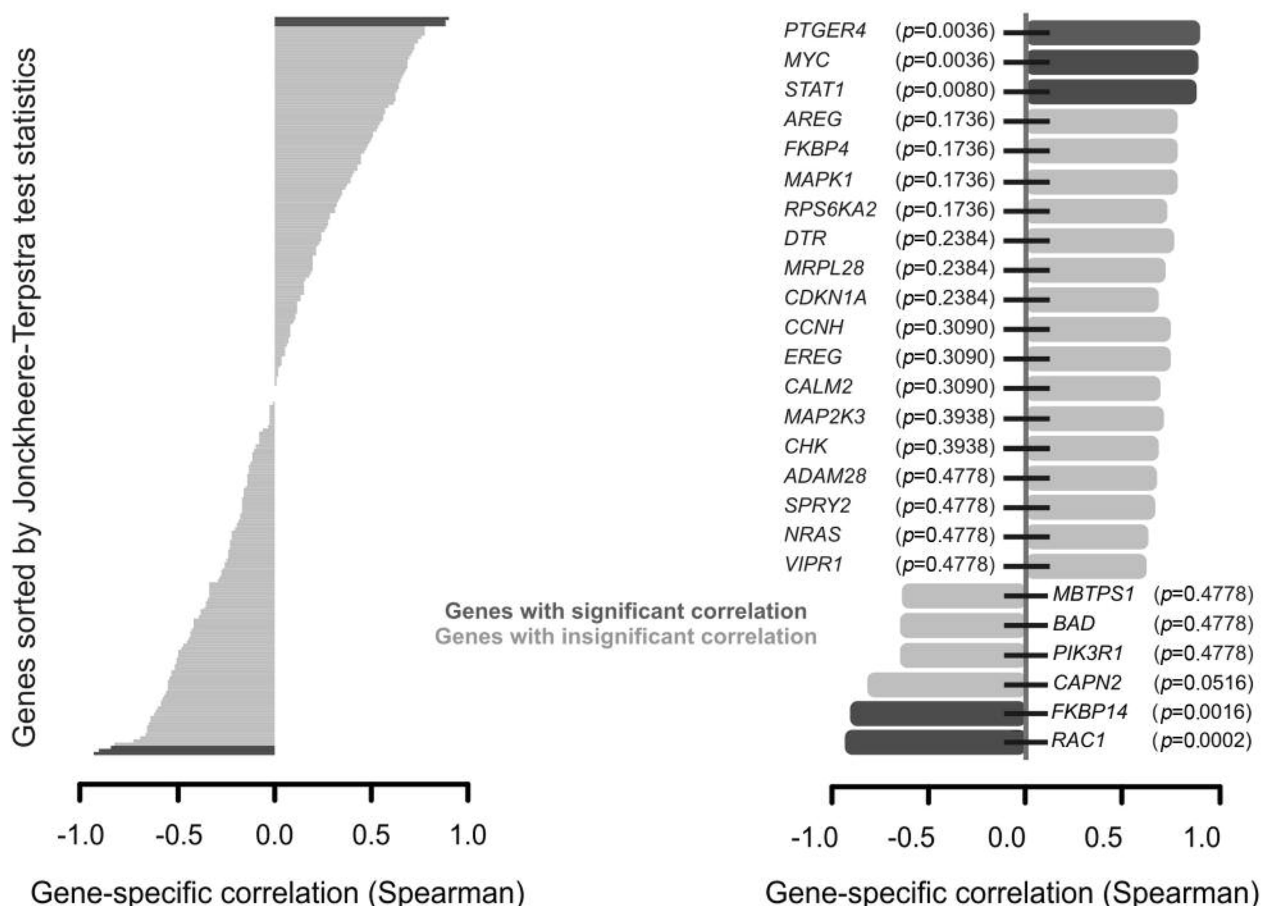

Figure 3. *Distribution of correlation of gene expression with three categories of cellular response to erlotinib (i.e. sensitivity, intermediate responsiveness, and resistance) across the entire source group of 244 genes (left panel). A subgroup of genes with the 30 highest Spearman correlation coefficients for gene expression and phenotype (i.e. biological response) is separately depicted (right panel). For abbreviations see Table I.*

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