Pathogenetic Pathways Leading to Glioblastoma Multiforme: Association between Gene Expressions and Resistance to Erlotinib

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Abstract. Background: The antiproliferative effects of erlotinib, an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, on human glioblastoma multiforme (GBM) cell lines in vitro and in vivo are widely variable and independent of EGFR baseline expression levels, indicating that more complex genetic signatures may form the molecular basis of GBM response to erlotinib. This study sought to determine which genes within two common genetic pathways of GBM pathogenesis, i.e. the primary and secondary pathways, may be involved in 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 prospectively selected 104 genes pertaining to the primary and secondary pathways of GBM pathogenesis identified two genes (IGF1, PIK3C2B) the expression of which significantly correlated with cellular resistance towards erlotinib. Conclusion: Among the genes constituting two common pathways of GBM pathogenesis, two candidate genes may confer GBM resistance towards erlotinib, suggesting that resistance towards this compound may be acquired during the natural evolution of GBM.

Erlotinib, an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, exerts widely variable antiproliferative effects on nine human glioblastoma multiforme (GBM) cell lines in vitro and in vivo (1). As those effects have been found to be independent of EGFR baseline expression levels, more complex signatures of gene expressions may ultimately underlie the molecular basis of the erlotinib-sensitive and erlotinib-resistant GBM phenotypes. A group of genes important to GBM pathogenesis constitutes two common genetic pathways, i.e. the primary and the secondary pathway (2-4). By examining a set of erlotinib-sensitive, intermediately responsive and erlotinib-resistant GBM cell lines, this study sought to determine candidate genes within these pathways for mediating sensitivity or resistance of human GBM towards erlotinib.

Materials and Methods

Glioblastoma cell lines. From nine established human GBM cell lines previously described (1), 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) (1) 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.

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 \textit{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. The raw microarray intensity values were preprocessed by performing background correction and cyclic loess normalization using the software R, version 2.5.0 (5). 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 104 genes pertaining to the primary and secondary pathways of GBM pathogenesis, 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 list obtained by statistical analysis was submitted to the Database for Annotation, Visualization and Integrated Discovery (DAVID) 2007 (http://david.abcc.ncifcrf.gov/).

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 3 μl of the obtained cDNA were added to the real-time PCR mix (17 μl) 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 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 two candidate gene targets with a product size ranging from 60-128 bases, the Universal ProbeLibrary Assay Design Center (Roche Applied Science) and Primer3 (http://primer3.sourceforge.net) were used. The primers (20-21-mers) were custom-synthesized by Sigma-Aldrich (Munich, Germany). The quantity of cDNA was calculated by normalizing the Ct values with those of previously chosen housekeeping genes (hypoxanthine-guanine phosphoribosyltransferase 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 prospectively selected 104 genes pertaining to the primary and secondary pathways of GBM pathogenesis identified two genes (\textit{IGF1}, \textit{PIK3C2B}) the expression of which significantly correlated with cellular response to erlotinib.
resistance towards erlotinib (Table I, Figure 1). These genes potentially represent therapeutic targets, interfering with which may enhance the efficacy of erlotinib against GBM. 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 2.

Deregulated expression of IGF1 has been observed in 17 different tumor entities (7). In GBM, overexpressed IGF1 participates in auto-, para- and endocrine growth-stimulating loops, inhibits apoptosis and assumes a critical role in transformation, proliferation and tumorigenicity (7, 8). On the other hand, PIK3C2B has recently been shown to be amplified and overexpressed in a subgroup of GBM (9), thereby contributing to aberrant signaling of the phosphoinositide-3-kinase (PIK3)/Akt, a pathway essential for GBM cell survival (10).

Gene expression of PIK3C2B was confirmed by qRT-PCR (data not shown). However, we were unable to specifically amplify IGF1 cDNA on repeated attempts with modified cycling conditions and different sets of gene-specific primers. While the nature of this difficulty remains undetermined, final proof of the involvement of IGF1 in modulating the cellular response of GBM towards erlotinib rests with causative confirmation experiments.

Each of the two genes identified as potentially conferring resistance to erlotinib may provide “escape from oncogene addiction”, i.e. a means for the GBM cell to circumvent...
otherwise critical EGFR inhibition and to maintain survival pathway signaling (11, 12). In view of the paramount importance of enhancing the efficacy of therapies for GBM, causative confirmation of the demonstrated statistical association between gene expressions and resistance towards erlotinib (e.g. by transfection experiments) is warranted. Moreover, this study provides a rational hypothesis regarding the extent of involvement of the primary and secondary pathway of GBM pathogenesis in conferring cellular resistance against erlotinib. This phenotype may be acquired during the natural evolution of GBM.

References
