Uniform *MDM2* Overexpression in a Panel of Glioblastoma Multiforme Cell Lines with Divergent *EGFR* and *p53* Expression Status

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Abstract. *Background: Overexpression and deletion mutation of the epidermal growth factor receptor (EGFR) gene, as well as murine double minute 2 (MDM2) overexpression have been linked to the absence of p53 gene mutations in human glioblastoma multiforme (GBM). Materials and Methods: EGFR and MDM2 messenger (m)RNA expression profiles and p53 status were examined by reverse transcription-polymerase chain rection (RT-PCR) and gene sequencing, respectively, in a set of human wild-type (wt) p53 GBM cell lines (U-87MG, U-87MG.wtEGFR and U-87MG.ΔEGFR) that exclusively differ in EGFR expression (endogenous wt EGFR expression, exogenous wt EGFR overexpression and exogenous 801-bp deletion-mutant [Δ] EGFR overexpression, respectively), as well as in two human mutant p53 GBM cell lines that differ approximately two-fold in endogenous wt EGFR mRNA expression. Results: Regardless of the underlying heterogeneity in EGFR mRNA expression and p53 status, MDM2 was similarly overexpressed among the cell lines. Conclusion: These data suggest that in human GBM (i) overexpression of wt or ΔEGFR and of MDM2 may constitute independent genetic events, (ii) overexpression of wt EGFR and mutation of p53 in GBM, although considered mutually exclusive in vivo, are not reciprocally prohibitive per se, and (iii) p53 mutations do not necessarily preclude MDM2 overexpression. In addition, this set of human GBM cell lines may constitute a suitable model for evaluating MDM2-targeted therapies in the context of various accompanying genetic alterations.*

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In glioblastoma multiforme (GBM), a devastating disease despite multidisciplinary therapeutic efforts, a large number of genetic alterations have been identified (23). Of those, some have been assigned to different pathways of GBM pathogenesis for which they are considered exclusive (22). Paramount examples are the amplification and deletion mutation of the *epidermal growth factor receptor* (*EGFR*) gene found in *de novo* (*i.e.,* primary) GBM, and the mutational inactivation of the tumor suppressor gene *p53* which characterizes the progressive lower-grade glioma (*i.e.,* secondary GBM) pathway. Overexpression of murine double minute 2 (MDM2), which under physiological conditions acts as a negative cellular regulator of p53 within an autoregulatory feedback loop (24), may abrogate wild-type (wt) p53 tumor suppressor function in primary GBM (4, 17), allowing those tumors which typically lack *p53* mutations to escape from p53-regulated growth control (20). Oncogenic effects independent of p53 have also been reported for both full-length and alternatively or aberrantly spliced *MDM2* transcripts (8), and MDM2 overexpression has been suggested as a prognostic marker for patients with GBM (5). In this report, a panel of human GBM cell lines with diverse constellations of *EGFR* and *p53* status and uniform levels of *MDM2* overexpression are described, suggesting that MDM2 may assume an autonomous role in GBM regardless of the status of genes that according to current concepts are functionally interrelated or mutually exclusive.

Materials and Methods

Glioblastoma cell lines. Characteristics and maintenance of the GBM cell lines U-87MG (HTB-14, obtained from the American Type Culture Collection, Rockville, MD, USA), U-87MG.wtEGFR and U-87MG.ΔEGFR (both kindly provided by Dr. H.-J. Su Huang, Ludwig Institute for Cancer Research, La Jolla, CA, USA), G-599GM and G-1163GM have been previously described (9, 11-13, 16, 19).

RNA extraction and complementary DNA synthesis. Isolation of total cellular RNAs from the above cell lines, treatment with RNase-free DNase I, and RT were performed according to standard protocols as described elsewhere (11). Commercially available total cellular RNA from human frontal lobe (Clontech, Mountain View, CA, USA) was reversely transcribed as a control for semi-quantitative assessment of *MDM2* mRNA expression by RT-PCR.

Polymerase chain reaction for assessment of EGFR and MDM2 mRNA expression levels. Amplification of cDNAs with gene-specific primers, ethidium bromide-stained electrophoresis and densitometrical assessment of the bands were carried out, as previously described (11).

DNA extraction and p53 DNA sequencing. Isolation of genomic DNAs from the cell lines and determination of *p53* mutational status by PCR-directed sequencing were performed, as detailed elsewhere (13).

Results and Discussion

As determined by densitometry of electrophoretically separated and ethidium bromide-stained bands, semi-quantitative RT-PCR employing two sets of primers for the *EGFR* target gene and the *β-actin* reference gene, respectively, indicated a four-fold expression of wt *EGFR* mRNA in U-87MG.wtEGFR as compared to the parental cell line U-87MG. After adjusting for divergent fragment sizes, Δ*EGFR* mRNA expression in U-87MG.ΔEGFR was six-fold in relation to wt *EGFR* mRNA in U-87MG. Lastly, wt *EGFR* mRNA expression in the primary GBM-derived cell line G-1163 was two-fold compared to the secondary GBM-derived cell line G-599GM and 0.6-fold compared to U-87MG (Figure 1). Whereas U-87MG and its two derivatives used in this study express wt *p53*, homozygous mutations of *p53* involving codon 249 and 195, respectively, have been found in G-599GM and G-1163GM (13, 18) and were confirmed in this study.

Notwithstanding these notable differences in *EGFR* and *p53* status, practically identical levels of *MDM2* over-expression were present among the cell lines (Figure 2). Overexpression was confirmed by subjecting commercially available total cellular RNA from human frontal lobe to the identical RT-PCR protocol (negative control), which yielded an extremely faint, hardly visible band (data not shown). This observation is surprising in that according to a widely accepted concept of glioblastoma pathogenesis, amplification and specific deletion mutation of the *EGFR* are largely exclusive to primary GBM, whereas inactivation of the tumor suppressor gene *p53* is essentially restricted to the secondary pathway. Therefore, *EGFR* amplification and mutation of *p53* are considered mutually exclusive (22). In primary GBM, the function of the wt *p53* tumor suppressor gene may be abolished by overexpression of MDM2 (4, 17).

Against this background, our results suggest that overexpression of *MDM2* in cell lines G-599GM and G-1163GM confers biological (*i.e.,* proliferative and/or anti-apoptotic) advantages that are independent of the activity of MDM2 on p53. Indeed, MDM2 has been shown to transform *p53* null cells (6), and the identification of oncogenic splice variants of human *MDM2* transcripts that lack the p53 binding site (21) provides further evidence of p53-independent functions of MDM2.

At least 40 alternatively and aberrantly spliced *MDM2* transcripts have been identified in tumors, and most of the variant transcripts lack a sequence that encodes at least part of the N-terminal p53 binding domain (1). *In vitro* binding assays have shown that the splice forms designated *MDM2*-A, -B, -C and -D yield products that lack the ability to bind to and inactivate p53 (21), and interestingly, these splice variants have also been detected in glioblastomas (15). Due to the C-terminal location [nucleotide positions 1374-1517 of 1526 bases for the full-length *MDM2* transcript (15)] of the primers used in our study, any of the above-mentioned splice forms may be present in the cell lines studied. Noteworthy, ultraviolet light-mediated *p53* induction failed to increase *MDM2* mRNA in U-87MG and U-87MG.ΔEGFR (11). By the same argument (*i.e.*, primer location), it can virtually be excluded that the *MDM2* mRNA that we amplified encodes a perinecrotic 60 kDa MDM2 isoform recently discovered within glioma spheroids and glioblastoma biopsy material (2). While this perinecrotic isoform is thought to have arisen *via* direct transcription rather than *via* a post-translational truncation event, it lacks the C-terminal, not the N-terminal domain. Further experimental work is required to elucidate the exact identities and functions of the *MDM2* mRNA species detected in this study.

The analysis of genomic imbalances of chromosomal segments presumably carrying glioma-associated genes in the primary GBM-derived cell line G-1163GM has demonstrated a gain of 7p12 harboring the *EGFR* locus while 12q14.3-15 encompassing *MDM2* remained unchanged (19). Applying this finding to the results of the current study suggests that in G-1163GM *EGFR* mRNA overexpression is, at least in part, due to gene amplification whereas *MDM2* is overexpressed at the mRNA level. Moreover, comparative genomic hybridisation has detected loss of 17p13 carrying the *p53* locus, which corresponds to the homozygous *p53* gene mutation demonstrated by DNA sequencing (13, 19).

Taken together, these data suggest that in human GBM overexpression of wt or Δ*EGFR* and of *MDM2* may constitute independent genetic events, overexpression of wt or Δ*EGFR* and *p53* mutations, though considered mutually exclusive *in vivo*, are not reciprocally prohibitive *per se*, and *p53* mutations do not necessarily preclude *MDM2* overexpression. The latter observation may reflect the fact that MDM2 has the potential to assume an oncogenic function in GBM cells independent of its inhibitory effect on p53.

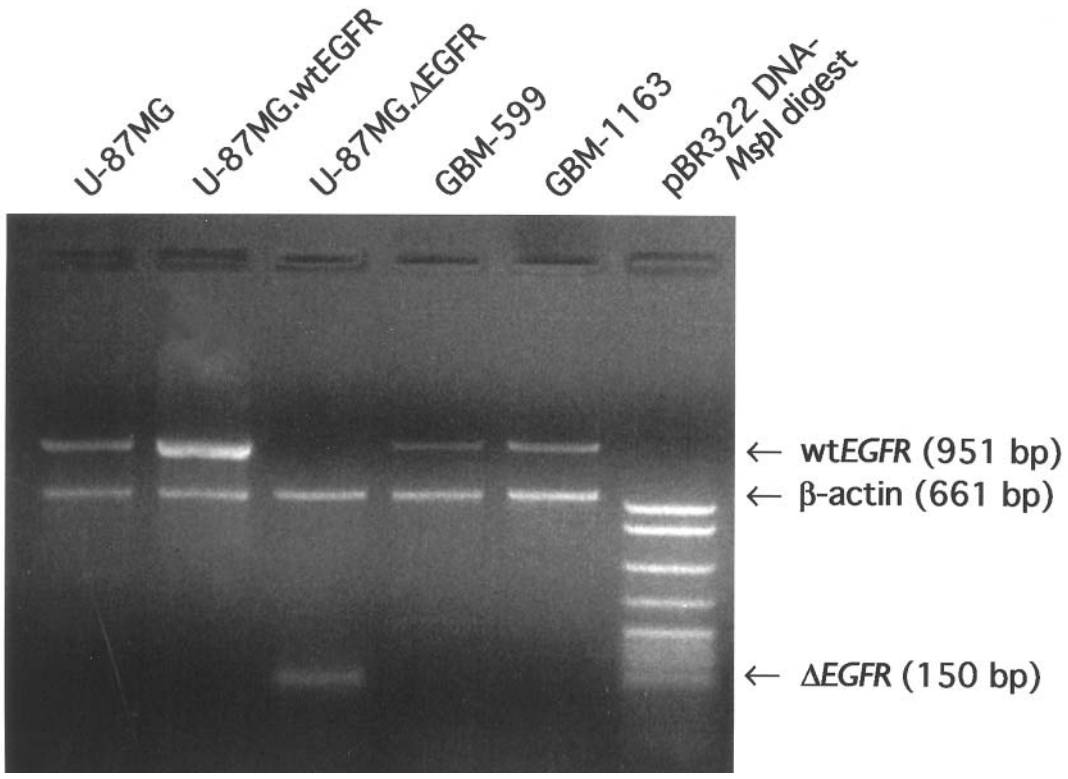

Figure 1. *Qualitative and semi-quantitative EGFR status in human GBM cell lines as determined by RT-PCR employing two sets of primers for the EGFR target gene and the β-actin reference gene, respectively. Expression of deletion-mutant EGFR is confirmed for U-87MG.ΔEGFR; the remaining cell lines express varying levels of wt EGFR.*

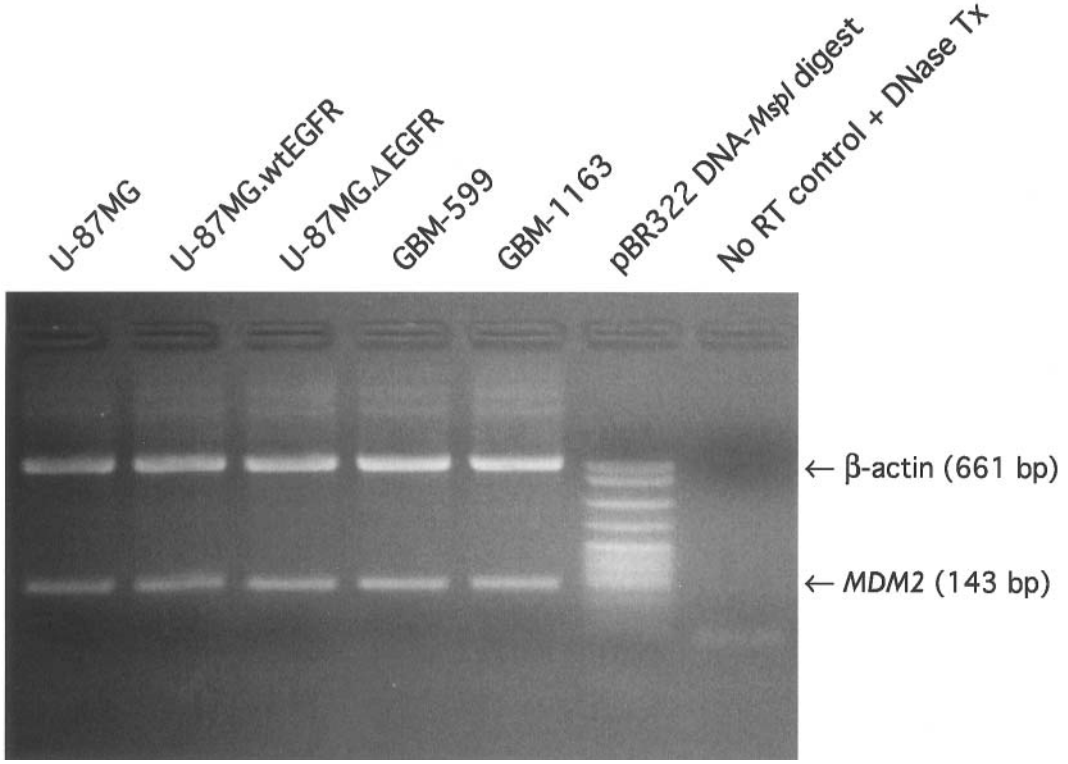

Figure 2. *Comparative MDM2 expression in human GBM cell lines (RT-PCR). MDM2 overexpression levels are constant regardless of EGFR and p53 status.*

Moreover, this set of human GBM cell lines may constitute a suitable model for evaluating MDM2-targeted therapies (3, 7, 18, 25) in the context of various accompanying genetic alterations, and more importantly, in concert with targeting other key molecules of glioblastoma pathogenesis (10, 14).

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