

Anthony Dipple *Carcinogenesis* Award

COMMENTARY

Genetics and new approaches to cancer therapy

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The past two decades have been golden years for the genetics of cancer. It has become clear through the work of countless laboratory groups that both inherited and sporadic cancers arise through defects or misregulations of their genomes. Several dozen dominantly acting oncogenes have been identified, shown to be deregulated in human tumors and to positively influence one or another aspect of tumor development or behavior in experimental systems. A smaller, but still substantial, number of recessively mutated tumor suppressor genes have also been identified which, in defective form, predispose to malignancy, both in people and in genetically manipulated rodent models. The cartography of the order, accumulation and interactions of genetic lesions during tumor initiation and progression has reasonable detail for many human tumor types. Such information is proving to be tremendously valuable in diagnosis and in grouping patients into prognostic categories. There is every reason to believe that the continued use of increasingly sophisticated genomic and proteomic technologies will add another order of magnitude to the precision of such conclusions.

One of the major reasons to have embarked on this sifting through the genomes of tumor cells has been the belief that tumor-specific defects will provide safer and more effective targets for therapy. While progress in this arena has been somewhat slower in pace, there are several examples now, such as drugs or antibodies targeting HER2, Bcr-Abl or the wild-type epidermal growth factor (EGF) receptor, to raise cautious optimism for the validity of this belief. It is now conceivable that future therapies will select combinations of drugs tailored to individual tumors and targeting with exquisite specificity different players in the same metabolic pathways or different pathways altogether. There are obvious advantages to such approaches: (i) the tumor-specificity of the targets should lead to a greater therapeutic window and less toxicity; (ii) the use of combination therapies may limit acquired resistance; and (iii) many of the defective pathways in tumor cells also affect drug or radiation sensitivities so that combined therapies using conventional and designed agents may be especially effective.

In this Commentary, I would like to use an example from our own work to illustrate how basic genetics can lead to the identification of such a tumor-specific target, understanding of its mechanism of action and informed creation of therapeutics specifically aimed at it. This work has focused on the role of

a specific mutated form of the EGF receptor in tumors of the central nervous system. Gliomas are the most common human brain tumors and their most malignant form, glioblastoma multiforme (GBM) (World Health Organization classification grade IV), still remains incurable despite intensive treatment regimens including surgery, radiotherapy and chemotherapy. Over the past 15 years, our group and several others have demonstrated that the malignant progression of gliomas involves a stepwise accumulation of genetic alterations that lead either to inactivation of tumor suppressor genes such as *TP53*, *CDKN2A*, *RB* and *PTEN*, or activation of oncogenes including the EGF receptor (*EGFR*), *CDK4*, *CDK6* and *MDM2* genes (1). We focus here on *EGFR* gene alterations as they are the most frequent dominantly acting oncogenic lesions in malignant gliomas.

The *EGFR* gene is often altered in malignant gliomas

The *EGFR* gene is commonly (40–60%) amplified in copy number up to hundreds of gene copies in GBMs (especially of the particularly aggressive *de novo* type) and some anaplastic astrocytomas (2), and is associated with high levels of *EGFR* mRNA or protein (3). In most cases, the gene is also rearranged during the process of amplification, which results in several classes of variant *EGFR* transcripts (4). The most common of these is a genomic deletion of exons 2–7, resulting in an in-frame deletion of 801 bp of the coding sequence, which generates a mutant receptor called de2-7 *EGFR*, Δ *EGFR* or *EGFRvIII* with a truncation of its extracellular domain (3,4). The Δ *EGFR* protein is detected in as many as 60% of GBMs and 20% of anaplastic astrocytomas (3,5) and has also been detected in cancers of the lung, breast and prostate (6–8), but not in normal tissues (3). *EGFR* gene amplification has been associated with a poor prognosis for patients with GBM (9) and patients with Δ *EGFR*-expressing GBMs have shorter life expectancies (10), suggesting that this tumor-specific genetic alteration may be involved with the aggressiveness of GBM tumors.

Δ *EGFR* is predominantly expressed on the cell surface and is constitutively active in glioma cells

The Δ *EGFR* gene was introduced into human GBM cells using retroviral-mediated gene transfer (11) where it was expressed at a level of $>10^6$ Δ *EGFR*/cell, similar to levels in human glioma biopsies (12,13). FACS analyses and immunofluorescence studies using monoclonal antibodies, which specifically recognized Δ *EGFR* demonstrated that Δ *EGFR* was expressed predominantly at the cell surface (11,13), similar to wild-type (wt) *EGFR* and to the expression seen in primary biopsy-derived glioma cells (12).

Unlike wt*EGFR*, Δ *EGFR* is constitutively phosphorylated at the C-terminal tyrosine residues although it is incapable of binding EGF or transforming growth factor (TGF)- α , natural ligands of wt*EGFR* *in vitro* (11,13–15), and also in glioma specimens (10). Furthermore, the receptor down-regulation

Abbreviations: CDDP, *cis*-diamminedichloroplatinum(II); EGF, epidermal growth factor; GBM, glioblastoma multiforme; PARP, poly(ADP-ribose) polymerase.

mediated for wtEGFR through internalization after ligand binding (16) was defective in Δ EGFR (13,15), suggesting that Δ EGFR may continuously transduce downstream signaling.

Phosphotyrosine residues in the carboxyl tail of wtEGFR provide sites for interaction with Src homology 2 (SH2) domain-containing adaptor molecules such as Shc and Grb2 (17). They recruit Ras to activated receptors, thereby increasing Ras activation, which initiates a cascade of mitogenic signaling pathways including activation of Ras-mitogen-activated protein kinase (MAPK) (18). Immunoprecipitation studies have shown that Δ EGFR is constitutively associated with phosphorylated Shc and Grb2 in several cell lines of different origins (15,19,20). Subsequent activation of Ras and MAPK was unique to glioma cells and not observed in other cell types expressing Δ EGFR but not wtEGFR (15,20). This suggests that the low level constitutive activation of Δ EGFR may cause coupling into unique pathways in these cells and may also point out an entry into interference therapies targeted at gliomas.

Enhancement of tumorigenicity by Δ EGFR

The constitutive activation of downstream signal transduction by Δ EGFR suggested a mechanism that might explain its association with highly aggressive disease. It was therefore puzzling that human glioma-derived U87MG. Δ EGFR cells grew similarly to parental U87MG cells in culture (11), while the receptor had only a modest *in vitro* transformation activity, determined by anchorage-independent growth in soft agar, in rodent fibroblasts (20,21). However, when inoculated into the flanks of nude mice, U87MG. Δ EGFR developed tumors much faster than parental cells and tumors initiated with a much smaller inoculum. Similarly, mice with intracerebral U87MG. Δ EGFR xenografts had significantly shorter life spans due to tumor growth, indicating that Δ EGFR confers enhanced tumorigenicity in glioma cells (11). We have made similar observations using several other human glioma cell lines and others have done the same with breast cancer cell lines (22). Consistent with our biochemical studies of activated signaling, the enhanced tumorigenicity is mediated through constitutively active Δ EGFR kinase activity and tyrosine autophosphorylation at the C-terminus, because a kinase defective mutation (DK) or mutation(s) of C-terminal tyrosine residue(s) (DY) resulted in ablation of the tumorigenic enhancement (13). Cell-mixing experiments demonstrated that a small number (as small as 0.001% of a mixed inoculum) of U87MG. Δ EGFR cells outgrew a large excess of the parental U87MG cells in intracerebral tumor formation in nude mice, suggesting that the growth advantage bestowed by Δ EGFR is intrinsic and exerted at the single-cell level (23).

Analysis of intracerebral xenografts for the proliferation markers, Ki67 antigen and BrdU-incorporation, revealed that the proliferation rate of U87MG. Δ EGFR-derived tumors was significantly higher than that for U87MG-, U87MG.wtEGFR- and U87MG.DK-derived tumors. In contrast, terminal deoxynucleotidyl transferase-mediated nick end-labeling (TUNEL) assays showed significantly reduced cell death in U87MG. Δ EGFR-derived tumors (23). The inhibition of spontaneous cell death in U87MG. Δ EGFR tumors was correlated with higher levels of the anti-apoptotic protein Bcl-X_L (23). These results clearly suggest that Δ EGFR confers enhanced tumorigenicity in glioma cells *in vivo* by both increasing proliferation and decreasing cell death, and that such growth-

promoting effects require its constitutively active kinase activity. Thus, not only the downstream pathways through which the receptor signals provide possible targets for intervention, its intrinsic enzymatic activity does as well.

Δ EGFR confers drug resistance to glioma cells

As Δ EGFR causes suppressed apoptosis in tumors by the up-regulated expression of the anti-apoptotic protein, Bcl-X_L, and many chemotherapeutic agents exert their antitumor effects by inducing apoptosis, we wondered whether Δ EGFR also conferred drug resistance through its inhibition of drug-induced apoptosis. Consistent with this notion, U87MG. Δ EGFR cells had significantly increased clonogenic survival to *cis*-diamminedichloroplatinum(II) (CDDP), taxol and vincristine, compared with parental U87MG, U87MG.DK or U87MG.wtEGFR cells (24). This resistance was associated with inhibition of drug-induced apoptosis as demonstrated by TUNEL assays (24).

Caspases are cysteine proteases, known to play a key role in the execution of apoptosis. CDDP-induced activation of the caspase 3-like effector proteases was significantly suppressed in U87MG. Δ EGFR cells. In addition, CDDP treatment resulted in cleavage of poly(ADP-ribose) polymerase (PARP), an intrinsic substrate of effector caspases, in U87MG, U87MG.wtEGFR and U87MG.DK cells but to only a minor extent in U87MG. Δ EGFR cells (24), mechanistically consistent with glioma cells expressing Δ EGFR being resistant to CDDP because of suppression of apoptosis.

Bcl-X_L expression levels were directly correlated with resistance to CDDP. Consistent with our findings for intracerebral xenografts, Bcl-X_L expression was high in U87MG. Δ EGFR cells prior to and during CDDP treatment, whereas it was at lower levels in the other receptor-containing cells and this further declined upon CDDP treatment. The direct role of Bcl-X_L expression on the drug resistance in U87MG. Δ EGFR cells was determined by overexpression of Bcl-X_L in parental U87MG cells (24). Such parental clones (overexpressing Bcl-X_L at levels comparable with those in U87MG. Δ EGFR cells) acquired resistance to CDDP-induced apoptosis and caspase activation (24).

Conversely, the Bcl-X_L expression level in U87MG. Δ EGFR cells was reduced when treated with a tyrosine kinase inhibitor, tyrphostin AG1478, which has been shown to inhibit constitutively active Δ EGFR kinase (25,26). As a result, treatment of U87MG. Δ EGFR cells with a combination of AG1478 and CDDP synergistically induced apoptosis, whereas treatment with either AG1478 or CDDP alone did not. This strongly suggested that Δ EGFR tyrosine kinase activity and Bcl-X_L expression are required for the drug-resistant phenotype (24). Moreover, the results suggest that targeting the receptor's tyrosine kinase activity might sensitize tumor cells to traditional chemotherapeutic drugs and so form the basis of one avenue of intervention.

Targeting Δ EGFR kinase with small molecule inhibitors

The molecular genetic and biological findings of a high incidence and expression of Δ EGFR in gliomas but not in normal tissues, cell surface expression and prominent growth advantages, strongly suggest that Δ EGFR could represent a novel molecular target for the treatment of the major subset of GBMs that express it. One potential therapeutic approach that exploits all of the biology uncovered about the receptor

is to utilize tyrosine kinase inhibitors against Δ EGFR kinase activity, as its constitutive activity is necessary for enhanced malignancy, increased proliferation, decreased apoptosis and drug resistance of glioma cells *in vitro* and *in vivo* (13,23,24). We have shown that a synthetic small compound, tyrphostin AG1478, has some selectivity for down-regulating tyrosine autophosphorylation of Δ EGFR compared with wtEGFR (25) and, as described above, AG1478 also synergistically enhances the cytotoxic effects of CDDP *in vitro*.

We therefore set out to test the hypothesis that inhibition of Δ EGFR constitutive tyrosine kinase activity could sensitize tumors to chemotherapeutic drugs. Animals bearing U87MG. Δ EGFR xenografts were treated with CDDP in combination with AG1478 or with each agent singly. Doses were chosen that caused little generalized toxicity or acute pathological changes in the liver and kidney. In comparison with the various control groups, the combination therapy significantly and synergistically suppressed the growth of subcutaneous tumors and extended the lifespan of animals with intracerebral tumors. In contrast, AG1478 did not have synergistic antitumor effects with CDDP for tumors derived from parental U87MG cells expressing wtEGFR or DK-EGFR, suggesting that AG1478 probably targets the constitutively active kinase of Δ EGFR *in vivo* and underscoring the potential specificity of such an approach (26). The pharmacokinetics of AG1478 in serum and organs in mice, in particular the level of its penetration through the blood-brain barrier, as well as appropriate delivery strategies need to be defined. Nonetheless, these studies provide pre-clinical proof-of-principle for one novel and informed therapeutic strategy against intractable GBMs, especially of the most aggressive *de novo* type and, perhaps, the other tumor types which have been shown to express the Δ EGFR.

Antibody therapy directed against Δ EGFR

An alternative approach to targeting the kinase activity of the receptor is to target the receptor itself. This is especially attractive due to the extracellular accessibility of the mutated portion of the receptor and its lengthy time on the cell surface. Monoclonal antibodies have been raised against the truncation epitope of Δ EGFR in its extracellular domain for the purpose of targeting it in glioma cells (14,27,28). Radiolabeled monoclonal antibodies specific to Δ EGFR have been shown to specifically and selectively localize to xenografts expressing Δ EGFR in nude mice (14,29). Because the receptor-bound monoclonal antibodies internalize, delivery of immunotoxins, such as antibody-conjugated *Pseudomonas* endotoxin A, to tumors with Δ EGFR has also yielded some favorable results (3,12,30,31).

We have developed a series of monoclonal antibodies raised against murine cells expressing the Δ EGFR (32,33). One of these, named mAb806, appears to be directed to a conformation-specific epitope outside of the truncation domain. We then determined the efficacy of mAb 806 on the growth of intracranial xenografted gliomas in nude mice. Systemic treatment with mAb 806 significantly reduced the volume of tumors and increased the survival of mice bearing xenografts of U87MG. Δ EGFR, LN-Z308. Δ EGFR or A1207. Δ EGFR gliomas, each of which expresses high levels of Δ EGFR. In contrast, mAb 806 treatment was ineffective with mice bearing the parental U87MG tumors which expressed low levels of endogenous wtEGFR or U87MG.DK tumors which expressed high levels of kinase-deficient Δ EGFR. A slight increase of

survival of mice xenografted with a wtEGFR overexpressing U87MG glioma (U87MG.wtEGFR) was effected by mAb 806 concordant with its weak cross-reactivity with such cells. Treatment of U87MG. Δ EGFR tumors in mice with mAb 806 caused decreases in both tumor growth and angiogenesis, as well as increased apoptosis. Mechanistically, *in vivo* mAb 806 treatment resulted in reduced phosphorylation of the constitutively active Δ EGFR and caused down-regulated expression of the apoptotic protector, Bcl-X_L. The data provide pre-clinical evidence that mAb 806 treatment may be another useful biotherapy for those tumors expressing Δ EGFR.

Conclusions

We are now seeing the beginnings of what has the potential to be a new age in cancer therapy. This has resulted from the joint interdisciplinary efforts of oncologists, surgeons, geneticists, molecular biologists and cancer researchers of many types. The long-term and substantial investment that has been made in understanding basic biology and the myriad ways that cancer subverts it should now pay huge dividends for patients. Specifically tailored and targeted therapies have the potential for greater efficacy and lesser toxicity than conventional approaches. Our studies here are but one example of the promise of these approaches. Similar successes with cancer prevention will be the ultimate payback. I have no doubt that such successes are coming.

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