A Novel EGFR Isoform Confers Increased Invasiveness to Cancer Cells

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Abstract

As a validated therapeutic target in several human cancers, the EGFR receptor (EGFR) provides a focus to gain deeper insights into cancer pathophysiology. In this study, we report the identification of a naturally occurring and widely expressed EGFR isoform termed EGFRvA, which substitutes a Ser/Thr-rich peptide for part of the carboxyl-terminal regulatory domain of the receptor. Intriguingly, EGFRvA expression relates more closely to histopathologic grade and poor prognosis in patients with glioma. Ectopic expression of EGFRvA in cancer cells conferred a higher invasive capacity than EGFR in vitro and in vivo. Mechanistically, EGFRvA stimulated expression of STAT3, which upregulated heparin-binding EGF (HB-EGF). Reciprocally, HB-EGF stimulated phosphorylation of EGFRvA at Y845 along with STAT3, generating a positive feedback loop that may reinforce invasive function. The significance of EGFRvA expression was reinforced by findings that it is attenuated by miR-542-5p, a microRNA that is a known tumor suppressor. Taken together, our findings define this newfound EGFR isoform as a key theranostic molecule. Cancer Res; 73(23): 7056–67. ©2013 AACR.

Introduction

As one member of the ErbB family, EGF receptor (EGFR) transferring extracellular signals to intracellular downstream factors plays a pivotal role in physiologic conditions such as embryonic development and in pathologic processes such as cancer cell proliferation, differentiation, invasion, and survival (1–3). Growth factors such as EGF induce EGFR dimerization, phosphorylation, and then activate various downstream signaling cascades, including AKT, mitogen-activated protein kinase, and STAT pathways. Abnormal expression of EGFR has been reported to be associated with a large number of human malignancies such as glioma, lung cancer, and colon cancer (4, 5). However, the correlation between EGFR expression and prognosis of patients with cancer still remains elusive.

Alternative splicing, known to affect more than half of all human genes, can lead to multiple mRNA variants and protein isoforms having related, distinct or even opposite functions (13). Protein isoforms serving as biomarkers or potential therapeutic targets for various types of cancer are of growing importance (14–16). The human EGFR gene spans approximately 188 kb and contains 28 exons (17). Several naturally occurring EGFR isoforms have been reported previously (17–19). However, most of them have not drawn great attention due to their tissue-restricted expression or infrequent occurrence. Moreover, most of them are soluble receptors, lacking an intracellular domain and difficult to mediate ligand-induced signal transductions. Some mutant splicing variants of EGFR have also been reported previously (20, 21). A well-recognized one is EGFRvIII. Nevertheless, the frequency of EGFRvIII is rather controversial (22–24). Thus, it seems necessary to perform more studies to understand the isoforms and variants of EGFR.

In this study, we identify a naturally occurring EGFR isoform, named as EGFRvA. Interestingly, EGFRvA expression is more significantly correlated with glioma grades and a poor prognosis of patients with glioma than EGFR expression. In in vitro and in vivo assays, EGFRvA promotes cancer cell migration and invasion, which are associated with the increased phosphorylation of STAT3 and the autocrine production of heparin-binding EGF (HB-EGF). Furthermore, our results showed that miR-542-5p, a microRNA (miRNA) acting as a tumor suppressor (25), can downregulate EGFRvA expression and inhibit cancer cell migration and invasion.
Identification of EGFRvA, a New Isoform of EGFR

Materials and Methods

Cell culture

The following cell lines were used in this study (all were from American Type Culture Collection, except Bel-7402 and U251MG): mouse embryonic fibroblast cell line, NIH/3T3; human epithelial carcinoma cell line, A431; human breast cancer cell line, MDA-MB-468; human glioblastoma-astrocytoma cell lines, U87MG and U251MG (Chinese Academy of Sciences, Beijing, China); human hepatocellular carcinomas cell line, Bel-7402 (Chinese Academy of Science); human prostate cancer cell line, PC-3; human lung adenocarcinoma cell line, NCI-H1299; human ovarian cancer cell line, SKOV-3; mouse hybridoma cell line producing M225 monoclonal antibody (mAb); and human embryonic kidney cell lines, 293 and 293FT. NIH/3T3 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Gibco) supplemented with 10% bovine calf serum (PAA Laboratories) and antibiotics (Gibco). Mouse hybridoma cells were cultured in RPMI-1640 medium with 2 mmol/L L-glutamine and supplemented with 15% horse serum. Other cells were cultured in DMEM supplemented with 10% FBS (Gibco) and antibiotics.

Clinical samples

Human cancer tissues were obtained along with written informed consent and pathology reports from hospitals and institutes as follows: Huashan Hospital (Shanghai, China; normal brain and glioma samples); Shanghai Chest Hospital (Shanghai, China; non–small cell lung cancer, NSCLC samples); and Qidong Liver Cancer Institute (Jiangsu, China; hepatocellular carcinoma samples). The use of all clinical materials within this study was reviewed and approved by the Institutional Ethics Review Committee of Shanghai Cancer Institute (Shanghai, China).

In vitro cell migration and invasion assays

Cells were suspended in serum-free medium or treated with serial dilutions of AG490 (or 10 μg/mL HB-EGF neutralizing antibody) for 30 minutes at room temperature. The cell suspension was then added to the upper chambers. Medium containing 10% FBS (or 5% FBS plus 10 μg/mL HB-EGF neutralizing antibody) was added to the lower chambers. Cells treated with 1% dimethyl sulfoxide (or mouse IgG) were used as negative controls. After incubation for several hours (see later) at 37 °C in a humid atmosphere with 5% CO2, cells were then fixed with 4% paraformaldehyde in PBS and stained with 1% crystal violet. The incubation time for the Transwell migration assay was 6 hours (U251MG), 12 hours (NIH/3T3), 16 hours (MDA-MB-468), or 24 hours (U87MG). The incubation time for the Transwell invasion assay was 12 hours (NIH/3T3 and U251MG) or 24 hours (U87MG and MDA-MB-468).

In vivo tumor invasion and metastasis analysis

U87MG transfectants (1 × 10⁶) were injected into the tail vein or the right hind flank of 6-week-old female nude mice. All animal experiments described in this study were approved by the Institutional Animal Care and Use Committee at Shanghai Cancer Institute.

Statistical analysis

Data were presented as mean ± SEM. Statistical significance was determined by paired or unpaired Student t test in cases of standardized expression data. One-way ANOVA was performed for multiple group comparisons and comparisons between two groups were conducted using the least significant difference method. The Kruskal–Wallis and Mann–Whitney U tests were used for nonparametric analyses of non-Gaussian data. A P value less than 0.05 was considered to be statistically significant.

Additional methods

Detail methods for reverse transcription PCR (RT-PCR), quantitative real-time PCR (qRT-PCR), lentivirus production and transduction, production and transduction of antibodies, Western blot analysis, immunocytochemistry/immunohistochemistry (IHC) and hematoxylin and eosin staining, immunoprecipitation, cell proliferation assay, cell viability and adhesion assay, micro-CT imaging, scoring system for spontaneous metastasis, microarray analysis, vector constructs, chromatin immunoprecipitation (ChIP), RNA interference, transfection, and luciferase assay are available in Supplementary Materials.

Results

Identification of a novel EGFR isoform, EGFRvA

When analyzing cDNA sequences of EGFR gene in the GenBank database, we noticed an EGFR variant transcript (GenBank accession number BC094761), which lacks the entire exon 4 and exon 28 but contains a portion of the intron between exon 27 and exon 28 (26). To verify this transcript, we applied long-distance RT-PCR to amplify its full-length cDNA in normal tissues, cancer cell lines, and cancer tissues (Supplementary Fig. S1A). Sequencing results showed that an exon 28 substitution did occur, whereas exon 4 was retained (Fig. 1A). This newfound transcript of EGFR (GenBank accession number GU255993), named as EGFRvA, is composed of 27 exons, encoding a novel protein bearing a carboxyl-terminal (C-terminal) Ser/Thr-rich peptide, which replaces a partial regulatory domain of EGFR spanning 1067E-1186A (Fig. 1B).

The presence of EGFRvA mRNA was further demonstrated by short-distance RT-PCR in seven cancer cell lines derived from different organs (Supplementary Fig. S1B). An increased EGFRvA expression was revealed in hepatocellular carcinoma and NSCLC tissues in comparison with corresponding adjacent noncancerous tissues by qRT-PCR (Fig. 1C). The results of qRT-PCR also showed that EGFRvA was highly expressed in placenta tissues and lowly expressed in other normal tissues (Supplementary Fig. S1C). To analyze the protein expression of EGFR and EGFRvA, specific mouse mAbs that can recognize them were generated (Supplementary Fig. S1D). EGFRvA and EGFR were both identified in most of the tested cancer cell lines (Fig. 1D). However, it is hard to detect EGFRvA separately from EGFR by a commercial antibody recognizing both of them because their molecular weights are too close. We then performed an immunoprecipitation in MD-MBA-468 by using a commercial antibody (Ab-3) against EGFR/EGFRvA.
and found EGFRvA was present in the precipitate, which further validated the endogenous expression of EGFRvA (Fig. 1E).

To further verify the expression of EGFRvA protein in cancer cell lines and tissues, we produced a specific polyclonal rabbit antibody against EGFRvA, which is available for immunocytochemistry/IHC. Our results showed that EGFRvA was detected in A431 and MDA-MB-468 cell lines by immunocytochemistry (Supplementary Fig. S1E). Taken together, we discovered a new EGFR isoform (EGFRvA), which was widely expressed in cancer cell lines and tissues.

The expression of EGFRvA is positively correlated with glioma grades and a poor prognosis of patients with glioma

The expression patterns of EGFR and EGFRvA were further explored in glioma tissues. Both isoforms were highly expressed in four of six glioma tissues in comparison with the corresponding adjacent noncancerous tissues, whereas EGFR but not EGFRvA was downregulated in the other two glioma tissues (Supplementary Fig. S2A). In addition, significant upregulation of EGFRvA rather than EGFR was revealed in the low-grade gliomas when compared with the normal brain tissues.
whereas upregulation of both isoforms was observed in the high-grade gliomas versus low-grade gliomas (Fig. 2A). EGFRvA can also be clearly detected in malignant glioma tissues by IHC. The IHC score was increased in high-grade glioma tissues compared with normal brain tissues and low-grade gliomas (Fig. 2B). Moreover, we found that only the expression of EGFRvA was significantly associated with a poor prognosis of patients with glioma (Fig. 2C and D; Supplementary Table S1). A poor prognosis in patients with high-grade gliomas was also revealed (Supplementary Fig. S2B). These results implied that EGFRvA played an important role in the progression of gliomas.

Figure 2. The expression of EGFRvA is associated with glioma grades and a poor prognosis of patients with glioma. A, left, protein expression of EGFR and EGFRvA in gliomas and normal brain tissues was detected by Western blot analysis. Right, relative protein levels (densitometry values normalized to GAPDH, glyceraldehyde-3-phosphate dehydrogenase) were quantified by ImageJ program. Data are mean ± SEM. B, IHC staining of EGFRvA in normal brain tissues and glioma tissues of different grades. A positive control (U87MG EGFRvA xenograft tissue) and a negative control (U87MG EGFR xenograft tissue) were used. C and D, Kaplan–Meier survival curve illustrating the impact of EGFR (C) and EGFRvA (D) expression on overall survival of patients with glioma. Western blot analysis of the protein levels of EGFR or EGFRvA (relative protein levels were quantified by ImageJ program, densitometry values normalized to GAPDH) in glioma tissues was used to classify glioma patients into two groups, above median or below median. P value was determined by log-rank test.
EGFRvA significantly promotes cell migration and invasion in vitro and in vivo

To investigate the biologic functions of EGFRvA, U87MG, and NIH/3T3 cell lines with stable expression of GFP, EGFR, or EGFRvA were established (Fig. 3A and Supplementary Fig. S1D). In growth curve assay (Supplementary Fig. S3A and S3B), both EGFR and EGFRvA significantly promoted cell proliferation in vitro. However, EGFRvA could not enhance tumor growth in vivo in comparison with GFP controls (data not shown). In Transwell migration and invasion assays, we observed that EGFRvA induced a 4-fold increase in cell migration and an 8-fold increase in cell invasion when compared with the GFP control, whereas EGFR only elicited cell migration and invasion rates to 2 to 3 times higher than that of the GFP control, respectively (Fig. 3B and Supplementary Fig. S3C). Similar results were also observed in U251MG and NIH/3T3 transfectants (Fig. 3C and Supplementary Fig. S3D), suggesting that EGFRvA conferred an increased motility and invasion to cells.

To verify the invasion-promoting capacity of EGFRvA in vivo, U87MG transfectants were injected intravenously into nude mice. U87MG EGFRvA–planting mice suffered from serious weight loss (Supplementary Fig. S3E) and 2 of them died on after injection day 16. The experimental metastases in lungs were observed by micro-CT scan on after injection day 18 (Supplementary Fig. S3F). Then the mice were sacrificed, and
we found that the most extensive nodules (Fig. 3D) and heaviest tumor burden (Fig. 3E) occurred in the U87MG EGFRvA group.

To further evaluate the tumor promoting properties of EGFRvA, tumor cells were subcutaneously inoculated into mice, which were sacrificed 8 weeks after inoculation. The most severe metastases to lungs were observed in the U87MG EGFRvA group (Supplementary Fig. S3G). Three of 7 mice in the U87MG EGFRvA group exhibited extrapulmonary metastases to the diaphragm, peritoneum lymph node, mesenteric lymph node, chest wall, and cavity (Fig. 3F and Supplementary Fig. S3H–S3M). In contrast, no extrapulmonary metastases were observed in the EGFR or GFP group (Fig. 3F). Altogether, these results indicated that EGFRvA significantly promoted cell invasiveness in vitro and in vivo.

EGFRvA overexpression leads to constitutive STAT3 activation and ensuing HB-EGF upregulation

To delineate the mechanisms underlying the invasion-promoting capacity of EGFRvA, several key molecules in the classical EGFR signaling pathways were examined. EGFRvA displayed a lower level of total tyrosine phosphorylation than EGFR (Fig. 4A), which may be ascribed to the lack of six tyrosine residues, including important autophosphorylation sites (Tyr1068, Tyr1086, Tyr1148, and Tyr1173). However, a significantly higher level of constitutive p-STAT3 was observed in the

Figure 4. EGFRvA overexpression causes constitutive STAT3 activation and ensuing HB-EGF upregulation. A, cells were cultured in complete medium or serum starved for 2 hours followed by the treatment of 100 ng/mL EGF for 8 minutes. The cell lysates were immunoprecipitated with Ab-3. The level of tyrosine phosphorylation in EGFR and EGFRvA was determined by Western blot analysis using phosphotyrosine-specific antibody, 4G10. B, key molecules of the EGFR signaling pathways were analyzed by Western blot analysis. C and D, examination of HB-EGF expression by qRT-PCR (C) and Western blot analysis (D). Data are mean ± SEM. E, Western blot analysis of the xenografts derived from the cell lines by using the indicated antibodies. Two distinct xenografts in each group were analyzed. F, glioma tissues were analyzed by Western blot analysis using the indicated antibodies. G, p-STAT3 level was correlated well with EGFRvA protein expression in glioma tissues (n = 52; nonparametric Spearman test).
cells overexpressing EGFRvA relative to EGFR (Fig. 4B and Supplementary Fig. 5A). No obvious differences were detected in the phosphorylation levels of AKT and extracellular signal-regulated kinase (ERK) between EGFRvA transfectants and EGFR transfectants (Fig. 4B and Supplementary Fig. 5A). There are substantial evidences suggesting that STAT3 activation can regulate the migration and invasion of cancer cells by augmenting the expression of matrix metalloproteinase-2 (MMP-2) and -9 (MMP-9; ref. 27). As expected, both MMP-2 and MMP-9 were expressed at higher levels in U87MG EGFRvA cells than in U87MG EGFR cells (Fig. 4B).

To further elucidate the molecular mechanisms underlying EGFRvA-mediated cell migration and invasion, cDNA array was performed. Compared with U87MG EGFR cells, U87MG EGFRvA cells contained higher expression of 86 genes and lower expression of 82 genes (Supplementary Tables S2 and S3). Among those upregulated genes, we noticed HB-EGF, encoding a well-known ligand of EGFR. qRT-PCR and Western blot analysis demonstrated that HB-EGF was expressed at a higher level in U87MG EGFRvA cells than in U87MG EGFR cells (Fig. 4C and D).

Like p-STAT3, MMP-2, and MMP-9, HB-EGF has been well demonstrated as an important player in cell migration and invasion (28). Elevated expression of these proteins in U87MG EGFRvA cells relative to U87MG EGFR cells was observed by immunofluorescence (Supplementary Fig. 4B). In addition, U87MG EGFRvA xenografts displayed highest expression of them (Fig. 4E). Furthermore, concordant to the EGFRvA expression, the expression of p-STAT3, MMP-2, MMP-9, and HB-EGF increased in the high-grade gliomas compared with the low-grade gliomas (Fig. 4F). Interestingly, there was a positive correlation between EGFRvA expression and STAT3 activation in glioma tissues (Fig. 4G).

**The positive feedback regulation between HB-EGF and p-STAT3 in EGFRvA-expressing cells**

To further investigate the relationship between HB-EGF and p-STAT3 in tumor cells with EGFRvA expression, we treated U87MG EGFR and U87MG EGFRvA cells with exogenous HB-EGF. Because EGFR Y845, Y992, and Y1045 were important tyrosine residues shared by EGFR and EGFRvA, their phosphorylations were examined. We found that Y845 but not Y992 and Y1045, could be phosphorylated in a time- and dose-dependent manner in both U87MG EGFR and U87MG EGFRvA cells treated with HB-EGF (Fig. 5A and Supplementary Fig. S5A). Interestingly, U87MG EGFRvA cells displayed a more robust phosphorylation at Y845 than U87MG EGFR cells (Fig. 5A). Although a time-related effect was also detected in ERK and AKT phosphorylation in both U87MG EGFR cells and U87MG EGFRvA cells upon HB-EGF treatment, no obvious differences were observed in the phosphorylation levels of ERK and AKT between the two cell lines (Fig. 5A). Intriguingly, upon HB-EGF treatment, U87MG EGFRvA cells but not U87MG EGFR cells displayed a time- and dose-response effect on STAT3 phosphorylation (Fig. 5A). STAT3 phosphorylation was also observed in U87MG cells incubated with the culture supernatant of U87MG EGFRvA cells rather than U87MG EGFR cells (Supplementary Fig. S5B). In addition, no p-STAT3 was found in U87MG cells treated with the culture supernatant of U87MG EGFRvA cells in the presence of HB-EGF neutralizing antibody (Supplementary Fig. S5B). These results further supported that the autocrine HB-EGF in U87MG EGFRvA cells could activate STAT3.

STAT3 is well known to induce gene expression and regulate important cellular processes through physically interacting and functionally cooperating with EGFR as well as JAK2 (29, 30). In all cell lysates, we found that EGFRvA-expressing U87MG cells had increased p-STAT3 and JAK2 kinase activity relative to EGFR-expressing U87MG cells upon HB-EGF treatment (Fig. 5B). Then, we sought to test whether EGFRvA could interact with STAT3 and JAK2. The results showed that greater abundance of p-STAT3 and STAT3 was coprecipitated with EGFRvA than with EGFR in U87MG transfectants upon HB-EGF stimulation (Fig. 5B). In addition, a higher level of p-JAK2 was found in the immunoprecipitated EGFRvA (relative to EGFR) complex, implying its involvement in the interaction between EGFRvA and STAT3 (Fig. 5B).

Interestingly, mRNA expression of HB-EGF was suppressed in both U87MG EGFRvA cells and U87MG EGFR cells transduced with STAT3-specific siRNA (Fig. 5C), suggesting that STAT3 can reciprocally regulate HB-EGF expression. By examining the sequences of human HB-EGF genomic structure, we identified nine putative sites conforming to the STAT3-binding consensus sequences TT(N4–6)AA (Supplementary Fig. S5C ref. 31) in the promoter region (−1000/+100, relative to the transcriptional start site). The results of the luciferase assay showed that forced STAT3 expression boosted HB-EGF promoter activity, which was further enhanced by the addition of interleukin (IL)-6, a factor known to activate STAT3 (Fig. 5D).

Furthermore, it was revealed by a ChIP assay that STAT3 could directly bind to the HB-EGF promoter (Supplementary Fig. S5D). In addition, the JAK2/STAT3 inhibitor AG490 could suppress HB-EGF expression in a dose-dependent manner in U87MG EGFRvA cells (Fig. 5E). Taken together, we disclosed that upregulated HB-EGF and activated p-STAT3 could positively regulate each other in EGFRvA-expressing cells.

**STAT3 and HB-EGF contribute to EGFRvA-mediated tumor invasiveness**

Furthermore, we found that the migratory and invasive potential of U87MG EGFRvA cells was gradually decreased by increasing concentration of AG490 (Fig. 6A), whereas cell viability and cell adhesion were almost unaffected by AG490 at a concentration equal to or less than 50 μmol/L (Supplementary Fig. S6A). In addition, the migratory and invasive capacities of U87MG EGFRvA cells but not the other two cell lines (U87MG GFP and U87MG EGFR) were robustly inhibited by STAT3-specific siRNA, indicating that STAT3 was required for EGFRvA-mediated tumor migration and invasion (Fig. 6B and C; Supplementary Fig. S6B).

To investigate the role of HB-EGF, we performed migration and invasion assays in the presence of a neutralizing antibody against HB-EGF. The results showed that the HB-EGF neutralizing antibody suppressed the migratory and invasive activities more effectively in EGFRvA transfectants than in EGFR transfectants (Fig. 6D). Moreover, the neutralizing antibody against
HB-EGF had an inhibitory effect on p-STAT3 in U87MG EGFRvA cells (Fig. 6E). These results further demonstrated that HB-EGF contributed to EGFRvA-mediated cell migration and invasion, which should be at least partially attributed to its effect on STAT3 activation.

EGFRvA is a target of miR-542-5p

Because EGFR and EGFRvA own different 3' untranslated region (UTR), we reasoned that EGFRvA could be differentially regulated by some miRNAs. We searched and found a likely candidate, miR-542-5p, which has been identified as a tumor suppressor (25). The reporters with wild-type or mutated 3' UTR of EGFRvA were constructed for luciferase assay (Fig. 7A). The results showed that miR-542-5p could reduce the expression of luciferase gene carrying wild-type 3'UTR of EGFRvA. This reduction was sequence-specific because the expression of the luciferase gene carrying mutated 3'UTR of EGFRvA or 3' UTR of EGFR was not affected (Fig. 7B). Because MDA-MB-468 contains the highest endogenous expression of EGFRvA among the tested cancer cell lines (Fig. 1D), it was used to elucidate the regulation of miR-542-5p on EGFRvA. Concordantly, an obvious reduction of endogenous EGFRvA but not EGFR was observed in MDA-MB-468 cells transfected with miR-542-5p mimics, whereas inhibition of miR-542-5p by its antagomiR increased the expression of EGFRvA rather than EGFR (Fig. 7C). In addition, in MDA-MB-468 cells, miR-542-5p could impede the cell migration and invasion as well as lamellipodia formation, and the impairments could be rescued...
Figure 6. STAT3 and HB-EGF are key players in EGFRvA-mediated cell migration and invasion. A, Transwell migration and invasion assays of U87MG EGFRvA cells treated with increasing concentrations of AG490. Data are mean ± SEM. B, cells were transfected with 100 nmol/L control siRNA or STAT3 siRNA for 48 hours and then subjected to Western blot analysis. C, U87MG transfectants pretreated with 100 nmol/L STAT3 siRNA or control siRNA for 48 hours were subjected to Transwell migration and invasion assays. Data are mean ± SEM. D, Transwell migration and invasion assays were performed on U87MG transfectants treated with HB-EGF neutralizing antibody or control IgG. Data are mean ± SEM. E, Western blot analysis of the cell lysates from U87MG transfectants with the treatment of HB-EGF neutralizing antibody (10 μg/mL) or control IgG for 24 hours.
by the ectopic overexpression of EGFRvA (Fig. 7D and E). We also analyzed the correlation between expression of miR-542-5p and EGFR or EGFRvA in glioma tissues. The results showed that the miR-542-5p expression was inversely correlated with the EGFRvA but not EGFR protein level in gliomas (Fig. 7F and G). All these data supported that EGFRvA expression could be specifically downregulated by miR-542-5p.

Discussion

In the present study, we discovered a new EGFR isoform, EGFRvA. Structurally, EGFRvA loses partial regulatory domain (1067E–1186A) of EGFR and carries one novel Ser/Thr-rich peptide at C-terminus. Accordingly, it is much likely that EGFRvA possesses some different biologic functions in comparison with EGFR.

Our results showed that EGFRvA is widely expressed in cancer cells and tissues, suggesting that it may play important roles in cancer development. Interestingly, compared with EGFR, the expression of EGFRvA was more significantly associated with glioma grades and a poor prognosis of patients with glioma. Furthermore, relative to EGFR, EGFRvA contributed more to the glioma cells motility and invasiveness in vitro and...
in vivo. Considering that EGFRvA may be a more important player than EGFR in the tumor progression of gliomas, it might be a better prognosis marker and a therapeutic target in gliomas. Moreover, it should be noted that the correlation between EGFR expression and prognosis of patients with cancer is controversial (6–9). We propose that the reasons for the inconsistency might be the existence of multiple EGFR isoforms or mutants and the current methods applied to determine the expression of EGFR can hardly distinguish them from each other.

We observed more robust JAK2/STAT3 activation as well as higher HB-EGF expression in the cancer cells expressing EGFRvA compared with EGFR in vitro and in vivo. Because upon HB-EGF stimulation, greater abundance of p-STAT3 and STAT3 was coprecipitated with EGFRvA than with EGFR in U87MG transfectants, we propose that EGFRvA, upon HB-EGF stimulation, can interact with greater abundance of p-STAT3 as well as STAT3 than EGFR. Interestingly, we also observed that STAT3 was able to inversely enhance HB-EGF expression via direct binding to its promoter, suggesting the positive feedback regulation between these two factors. HB-EGF plays a pivotal role in tumor progression by contributing to cell adhesion, cell motility, and angiogenesis (28, 32). In our study, blockade of HB-EGF with its neutralizing antibody and down-regulation of STAT3 by AG490 or specific siRNA significantly suppressed the EGFRvA-mediated invasiveness. Taken together, we propose that HB-EGF contributes to EGFRvA-elicited tumor invasiveness through STAT3 signaling pathway by an autocrine mechanism.

Then, we attempted to understand how STAT3 is activated by EGFRvA. Notably, we observed that the HB-EGF stimulation induced higher level of Y845 phosphorylation in EGFRvA than in EGFR. The phosphorylation of Y845 was involved in tumor progression (33). Recently, it was also reported that the phosphorylation of Y845 in EGFR was proposed as part of a self-sustained signal propagation and amplification process (34). Thus, the higher level of Y845 phosphorylation in EGFRvA (relative to EGFR) is a likely explanation for the activated JAK2/STAT3 as well as the elevated tumor invasiveness. Besides, a previous study reported that the EGFR sequence between Y954 and Y974 was essential for STAT activation, whereas the EGFR sequence between Y1114 and E1172 negatively regulated STAT activities (35). So the increased p-STAT3 caused by EGFRvA may be due to the loss of the negative regulation region of EGFR (E1067–A1186). Another possible explanation for the potent activation of STAT3 by EGFRvA is the presence of the Ser/Thr-rich peptide located in the C-terminus of EGFRvA.

The expression pattern of EGFRvA is different from that of EGFR, which might be ascribed to their different 3’UTR. Intriguingly, miR-542-5p, previously identified as a tumor suppressor of gliomas (25), was disclosed as a regulator of EGFRvA expression via directly targeting its 3’UTR. Several miRNAs have been demonstrated as regulators of EGFR expression, including miR-542-5p (36–38). However, we did not observe that miR-542-5p could downregulate EGFR expression in MDA-MB-468 cells. The reason for this discrepancy may be that the primers and antibodies previously used could not distinguish EGFRvA from EGFR.

Nowadays, more and more knowledge about EGFR has been updated, such as its oncogenesis in the presence of Kras mutation and its tumor suppression activities (39, 40). Undoubtedly, our discovery of EGFRvA further expands the knowledge about EGFR. Because EGFRvA can potently promote cancer cell invasion and its expression has a positive correlation with a poor prognosis of patients with glioma, it might be a prognosis biomarker and a therapeutic target for cancer treatment.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: M. Zhou, H. Wang, X. Pan, Z. Li
Development of methodology: M. Zhou, H. Wang, X. Pan, Z. Li
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Zhou, X. Luo, S. Lu, M. Yao, Y. Mao
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Zhou, H. Wang, X. Luo, X. Pan, Z. Li
Writing, review, and/or revision of the manuscript: M. Zhou, H. Wang, X. Pan, L. Zhang, J. Gu, C. Li, Z. Li
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B. Shi, H. Jiang, K. Li, H.-M. Wang, H. Gao, H.-Y. Wang, S. Yang
Study supervision: Z. Li

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