



The epidermal growth factor receptor variant III (EGFRvIII): where wild things are altered

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The epidermal growth factor receptor (EGFR) is overexpressed in a variety of human epithelial tumors, often as a consequence of gene amplification. Tumors with *EGFR* gene amplification frequently contain *EGFR* gene rearrangements, with the most common extracellular domain mutation being EGFRvIII. This mutation leads to a deletion of exons 2–7 of the *EGFR* gene and renders the mutant receptor incapable of binding any known ligand. Despite this, EGFRvIII displays low-level constitutive signaling that is augmented by reduced internalization and downregulation. Aberrant EGFRvIII signaling has been shown to be important in driving tumor progression and often correlates with poor prognosis. It is clear that EGFRvIII is expressed in a considerable proportion of patients with glioblastoma multiforme (GBM). The presence of EGFRvIII in other tumor types, however, remains controversial. In this review, we critically analyze the evidence for the expression of EGFRvIII in a range of tumor types and discuss recent findings pertinent to its function and biology in GBM.

Introduction

The epidermal growth factor receptor (EGFR) is a pivotal regulator of normal cellular growth in tissues of epithelial origin. Dysregulated EGFR signaling (resulting from mechanisms such as cell-surface overexpression, autocrine activation and *EGFR* gene mutation) contributes to the formation of many epithelial malignancies in humans [1,2]. Where dysregulation is the result of cell-surface EGFR overexpression, there is often associated gene amplification [1] and/or mutation [3,4]. Although several EGFR mutations have been

described, the most common extracellular mutation is EGFRvIII (also known as de2-7EGFR and Δ EGFR). EGFRvIII is a tumor-specific mutation that results from in-frame deletion of 801 base pairs spanning exons 2–7 of the coding sequence [5–7]. This deletion removes 267 amino acids from the extracellular domain, creating a junction site between exons 1 and 8 and a new glycine residue (Fig. 1) [8,9]. EGFRvIII has a molecular mass of approximately 145 kDa [10] and has similarities to the v-ErbB transforming protein of avian erythroblasto-

Abbreviations

AMPK, AMP-activated protein kinase; CDK, cyclin-dependent kinase; DOCK180, dedicator of cytokinesis 1; EGFR, epidermal growth factor receptor; FDG, fluorodeoxyglucose; FOX, forkhead; GBM, glioblastoma multiforme; HGF, hepatocyte growth factor; IHC, immunohistochemistry; IL-8, interleukin-8; LIF, leukemia inhibitory factor; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; mTORC2, CREB-regulated transcription co-activator 2; NF- κ B, nuclear factor κ B; NSCLC, non-small-cell lung cancer; PDGFR β , platelet-derived growth factor receptor β ; PI3K, phosphatidylinositol 3-kinase; RTK, receptor tyrosine kinase; SFKs, Src family kinases; Stat3, signal transducer and activator of transcription 3; VEGF, vascular endothelial growth factor; VEGFR2, VEGF receptor 2; WB, western blotting; wt, wild-type.

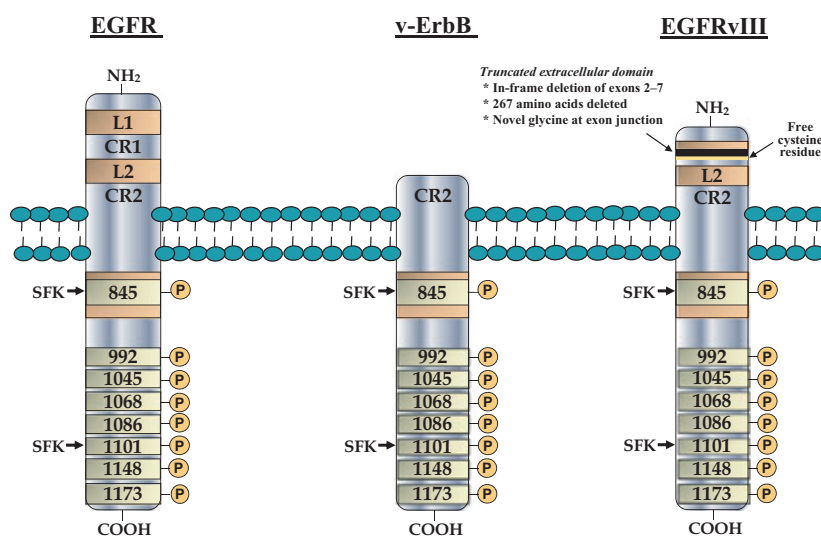


Fig. 1. Schematic structure of EGFRvIII. Compared with the epidermal growth factor receptor (EGFR), EGFRvIII has an in-frame deletion of exons 2–7, resulting in a shorter extracellular domain and the insertion of a novel glycine residue at the junction site. The resultant truncated receptor is constitutively active and cannot bind any known ligand. The juxtamembrane and intracellular regions of the EGFR are conserved in EGFRvIII. The domains of EGFR are shown, labeled domains L1 (also known as domain 1), CR1 (domain 2), L2 (domain 3) and CR2 (domain 4) respectively. COOH, carboxy terminus; NH₂, amino terminus; SFK, Src family kinase.

sis virus, which also is an EGFR-related auto-activating oncogene created by a large extracellular deletion [11].

Detection and analysis of EGFRvIII

The EGFRvIII literature must be approached with an appreciation of the technical limitations. Most reports on EGFRvIII are based on transfected cell lines [4,10,12–112]. This has been necessary because, when traditional cell lines are grown *in vitro*, they lose *EGFR* gene amplification and mutation [113–117]. This consistent negative *in vitro* selection for EGFRvIII [9,116–118] is poorly understood and raises concerns about data based solely on transfected cell lines. More recently, researchers have been able to naturally maintain EGFRvIII expression if the lines are generated directly from EGFRvIII-positive tumors and propagated *in vivo* [9,116–118], as neurospheres in three-dimensional cultures [119,120] in specialized media [121].

There are also challenges in the detection of EGFRvIII in human tissues. Several monoclonal antibodies specific for EGFRvIII have been described [7,12,14,19,27,30,40,47,63,73,122–125], but their availability to researchers is restricted by patenting and licensing issues [126]. Furthermore, there are concerns about whether these antibodies are indeed specific for EGFRvIII. Limited data suggest that the false positive rate of some of these antibodies ranges from 4% to 100% [7,66,115,126–129].

The lack of readily available antibodies against EGFRvIII has encouraged some research groups to use PCR to search for the presence of EGFRvIII in human tumors. This approach is also problematic as the presence of EGFRvIII transcripts does not necessarily correlate with protein levels of EGFRvIII that are functionally relevant. Between 0% and 63% of tumors that are positive for EGFRvIII by PCR have been reported to have no associated EGFRvIII protein [7,115,130–132]. To obtain the most reliable and valid data, future studies should use two complementary techniques to detect EGFRvIII: a PCR-based technique for detecting the presence of EGFRvIII transcripts and at least one protein-based method. Immunohistochemistry (IHC) or fluorescence activated cell sorting are preferred because western blotting (WB) can detect trivial amounts of protein; very limited data suggest that 69% of positive WB results are not confirmed by another protein detection technique [131]. Using the double-assay approach, EGFRvIII would be considered to be present only if there were concordant positive results using both methods [126].

Finally, the overwhelming evidence indicates that EGFRvIII is a tumor-specific receptor, with almost all published studies showing that normal tissues are devoid of EGFRvIII [5,7,9,103,115,129–131,133–145]. Only one study found EGFRvIII expression in normal lung tissue, using IHC [144]. We consider that this finding is an artifact of the IHC because the result has not been reproduced.

EGFRvIII expression in glioblastoma multiforme (GBM)

Most EGFRvIII research has been performed on high-grade brain tumors such as GBM, the most common and lethal malignant neoplasm of the brain in adults. GBM is increasing in incidence, is difficult to treat and has a very poor prognosis – the 1-year survival rate for patients who are newly diagnosed with GBM is 18% [9,17,146–149]. Amplification of the *EGFR* gene and the subsequent overexpression of the EGFR protein is the most common genetic alteration in GBM, occurring at a frequency of approximately 34–63% [137,140,142,150–153]. Of these cases, 63–75% also carry rearrangements of the *EGFR* gene, resulting in tumors expressing both wild-type (wt) and mutated EGFR [135,141,143,150,154,155]. The most common mutation in GBM is EGFRvIII, which occurs at an overall frequency of 25–64% when assessed by multiple techniques in the same tumor (Table 1) [5,7,9,103,129–131,133–135,137,138,140–143]. EGFRvIII expression in GBM is almost always associated with amplification and co-expression of the wt *EGFR* gene [129,134,145,150]. Some studies have suggested that EGFRvIII expression may be associated with a poor prognosis in patients with GBM [131,140]. One of the larger reported studies analyzed the prognostic value of *EGFR* gene amplification and mutation in 87 adult patients newly diagnosed with GBM. This study demonstrated that EGFRvIII expression, in combination with *EGFR* gene amplification, was an independent and significant negative prognostic factor for survival, even after adjusting for established prognostic factors [140]. Not all studies, however, have shown an association between EGFRvIII and patient outcomes [128,129,145].

EGFRvIII expression in other human malignancies

EGFRvIII expression has also been reported in malignancies outside the central nervous system (Table 1). Given some of the technical problems with EGFRvIII detection, especially those that occur when using polyclonal antibodies to detect EGFRvIII [127,139,156], several of these reports remain controversial. Our overview (Table 1) is restricted to studies that were conducted using primary tumor tissue from adults and distinguishes between frequencies defined using only one technique (left half of Table 1) and those defined using two or more complementary techniques (right half of Table 1), with the latter group likely to be more reliable. Many of the data are limited and con-

flicting. For example, Moscatello *et al.* [138] reported EGFRvIII expression in 24 of 32 (75%) ovarian carcinoma samples examined by WB. Subsequently, several large independent studies using more reliable methods for EGFRvIII detection were performed to verify EGFRvIII expression in ovarian cancer. Importantly, EGFRvIII was not detected at the mRNA or protein level in any of the ovarian carcinoma tissue samples or cell lines tested [5,114,157–159], indicating that EGFRvIII is not expressed in ovarian cancer (Table 1). Similarly, EGFRvIII is also not expressed in colorectal cancer, with three [5,65,160] of four studies [5,65,160,161] confirming the lack of EGFRvIII expression at both the mRNA level and the protein level. For all other tumor types that have been tested, the data remain preliminary, especially given that most of the data are derived from solitary studies that used only one detection technique. We await corroborative data for these tumor studies, which hopefully will use more reliable methods and reagents for EGFRvIII detection at the mRNA and protein levels.

EGFRvIII expression in breast carcinoma

The proportion of breast carcinomas that express EGFRvIII remains unresolved. Some studies have reported EGFRvIII expression in 20–78% of breast tumor tissues [7,115,138,162], whereas others have found negligible expression in tumor tissues (0–4%) [114,128,163] (Table 1). In a study conducted by Ge *et al.* [115], using laser capture microdissection and reverse transcription PCR (RT-PCR) on tissue from 28 patients with breast cancer, approximately 68% (19 of 28) of primary invasive breast cancers expressed high levels of EGFRvIII mRNA. The tumors from 11 of these patients were also investigated by IHC using the EGFRvIII-specific monoclonal antibody Ab-18 (also known as DH8.3; no longer available commercially), and 10 were found to also have EGFRvIII expression. No detectable levels of EGFRvIII were observed in normal breast tissue. Furthermore, 57% of the infiltrating breast carcinoma samples (16 of 28) were shown to co-express EGFRvIII and wt EGFR [115]. Using the monoclonal antibodies L8A4 and Y10, Wikstrand *et al.* [7] found that breast cancers express EGFRvIII in 27% of cases. By contrast, Rae *et al.* [114] showed that 55 breast cancer cell lines and 170 primary breast cancers were devoid of EGFRvIII mRNA, as determined by RT-PCR, and Nieto *et al.* [128] found that only 4% of patients with advanced breast carcinoma (9 of 225), whose samples were screened by IHC using the EGFRvIII-specific antibody DH8.3, showed evidence of EGFRvIII expression.

Table 1. EGFRvIII expression in human malignancies. IHC, immunohistochemistry; SB, Southern blotting; WB, western blotting; N/D, not determined; HNSCC, head and neck squamous cell carcinoma; AD, adenocarcinoma; LA, large cell carcinoma; SCC, squamous cell carcinoma; PNST, peripheral nerve sheath tumor; MFH, malignant fibrous histiocytoma.

Tumor type	Single technique used			Multiple complementary techniques used		
	EGFRvIII frequency (%)	Technique	Studies	EGFRvIII frequency (%)	Techniques	Studies
Brain (glioma)						
Grade IV (glioblastoma)	0–81	IHC	[5,7,126,129,134,137,140,180,224–227]	64	RT-PCR, IHC	[130]
	26–55	PCR	[5,118,126,133,228]	25	RT-PCR, IHC, WB	[131]
	17	SB	[141]			
Grade III	14–25	IHC	[7, 129]		N/D	
	12–40	PCR	[133,228]			
Grade II	0	PCR	[228]		N/D	
	86	WB	[138]		N/D	
Brain (oligodendroglia) ^a						
Grade II	0	IHC	[229]			
Grade III	0–7	IHC	[145,229]			
Bladder	0–50	IHC	[5, 66]	0	IHC, PCR, WB	[66]
Breast	0–32	IHC	[5,128,162]	20–36	IHC, RT-PCR	[7, 115]
	0–68	PCR	[114,115,163,165,230]		N/D	
	78	WB	[138]			
Colorectal	0–34	IHC	[5,161]		N/D	
	0	PCR	[65,160]			
	0	WB	[160]			
Esophageal	0	IHC	[5]		N/D	
HNSCC	0–42	IHC	[5, 72]	33 ^b	IHC, PCR	[72]
Lung						
AD	0–41	IHC	[5,136,144,164]		N/D	
	0–1	PCR	[132,166–168]			
LA	0–33 ^b	IHC	[5,144,164]		N/D	
	14	PCR	[165]			
SCC	0–46	IHC	[5,136,144,164]	5	IHC, PCR	[132]
	0–8	PCR	[165,167,168]		N/D	
Others ^c	0–100 ^b	PCR	[136,165]			
Melanoma	0	IHC	[5]		N/D	
Ovarian	0	IHC	[5,157]	0	IHC, PCR	[157,158]
	0–75	PCR	[138,157]	0	PCR, WB	[159]
PNST	0	IHC	[231]		N/D	
Prostate	100	IHC	[139,172]		N/D	
Sarcoma					N/D	
Leiomyosarcoma	0	IHC	[5]			
Synovial sarcoma	0	IHC	[5,231]			

Table 1. (Continued).

Tumor type	Single technique used			Multiple complementary techniques used		
	EGFRvIII frequency (%)	Technique	Studies	EGFRvIII frequency (%)	Techniques	Studies
MFH	0	IHC	[5]			
Osteosarcoma	0	PCR	[232]			
Testicular seminoma	0	IHC	[5]		N/D	
Thyroid					N/D	
Papillary	75	IHC	[156]			
Follicular	43	IHC	[156]			
Medullary	0	IHC	[156]			

^a Either pure oligodendrogliomas or mixed oligoastrocytomas. ^b Total sample size < 5 samples. ^c Includes adenocarcinomas.

More recently, Yu *et al.* [162] examined 182 normal breast tissue and breast carcinoma samples, including lymph node tissue taken from patients with metastatic breast carcinoma. EGFRvIII expression was assessed by IHC using the EGFRvIII-specific antibody DH8.3 and was found in 18% of ductal carcinomas *in situ*, 32% of primary invasive breast carcinomas and 54% of lymph node tissue samples taken from patients with metastatic disease [162]. No EGFRvIII was detected in normal breast tissues, consistent with previously reported findings [115]. Collectively, these reports demonstrate that EGFRvIII expression is correlated with disease progression in breast cancer and may be involved in tumor metastasis.

EGFRvIII expression in lung carcinomas

The proportion of non-small-cell lung cancers (NSCLCs) that express EGFRvIII is also controversial (Table 1). EGFRvIII expression was found in 16–39% of NSCLC samples in two studies using either the polyclonal antibody ZMD.82 or the monoclonal antibody G100 [136,164]. Both of these antibodies, however, non-specifically stained non-malignant cells and tissues such as type II pneumocytes, normal ciliated bronchial epithelium and submucosal bronchial glands. A consensus view has emerged that the proportion of NSCLCs that express EGFRvIII is much lower than is reported in these studies and that the expression of EGFRvIII seems to be strongly influenced by the histological subtype of NSCLC and inversely correlated with the presence of kinase domain mutations in exons 18–21 of wt *EGFR*. Several studies have shown that tumors with an adenocarcinoma subtype frequently express EGFR with kinase mutations (11–52% of

tumors) [165–167], but they are universally devoid of the EGFRvIII mutation [132,165–167] despite the significant correlation between receptor tyrosine kinase (RTK) mutations and wt EGFR overexpression. By contrast, kinase mutations are less frequent in tumors of a non-adenocarcinoma subtype (14%) [165], but a small proportion of these tumors (0–8%) reproducibly express EGFRvIII [132,165,167,168].

EGFRvIII confers enhanced tumorigenicity through multiple mechanisms and pathways

EGFRvIII has been shown to increase the tumorigenicity of cells of diverse origins, including those in GBM [4,7,17,21,34,51,82,115,146,153,169–184], NIH3T3 murine fibroblasts [10,16,123] and in breast [162], lung [132] and ovarian [74] cancers. Given the consistency of the results across cell types, we focus predominantly on the data for GBM. GBM is a highly proliferative, vascular and locally invasive tumor [185–187]. Consistent with these clinicopathological features, EGFRvIII-transfected GBM cell lines show increased rates of proliferation [146,173], an increased ability to form tumor xenografts [4,146,183], reduced apoptosis [146], increased angiogenesis [184] and increased invasiveness [188–192] compared with matched parental cell lines.

These pro-tumorigenic effects of EGFRvIII seem to be mediated by several key signaling pathways downstream of EGFRvIII, including phosphatidylinositol 3-kinase (PI3K)/Akt [7,51,179–182], Ras/Raf/mitogen-activated protein kinase (MAPK) [169], signal transducer and activator of transcription 3 (Stat3) [34,82,115,170–172] and nuclear factor κ B (NF- κ B) [184] (Fig. 2). Interestingly, the growth advantage

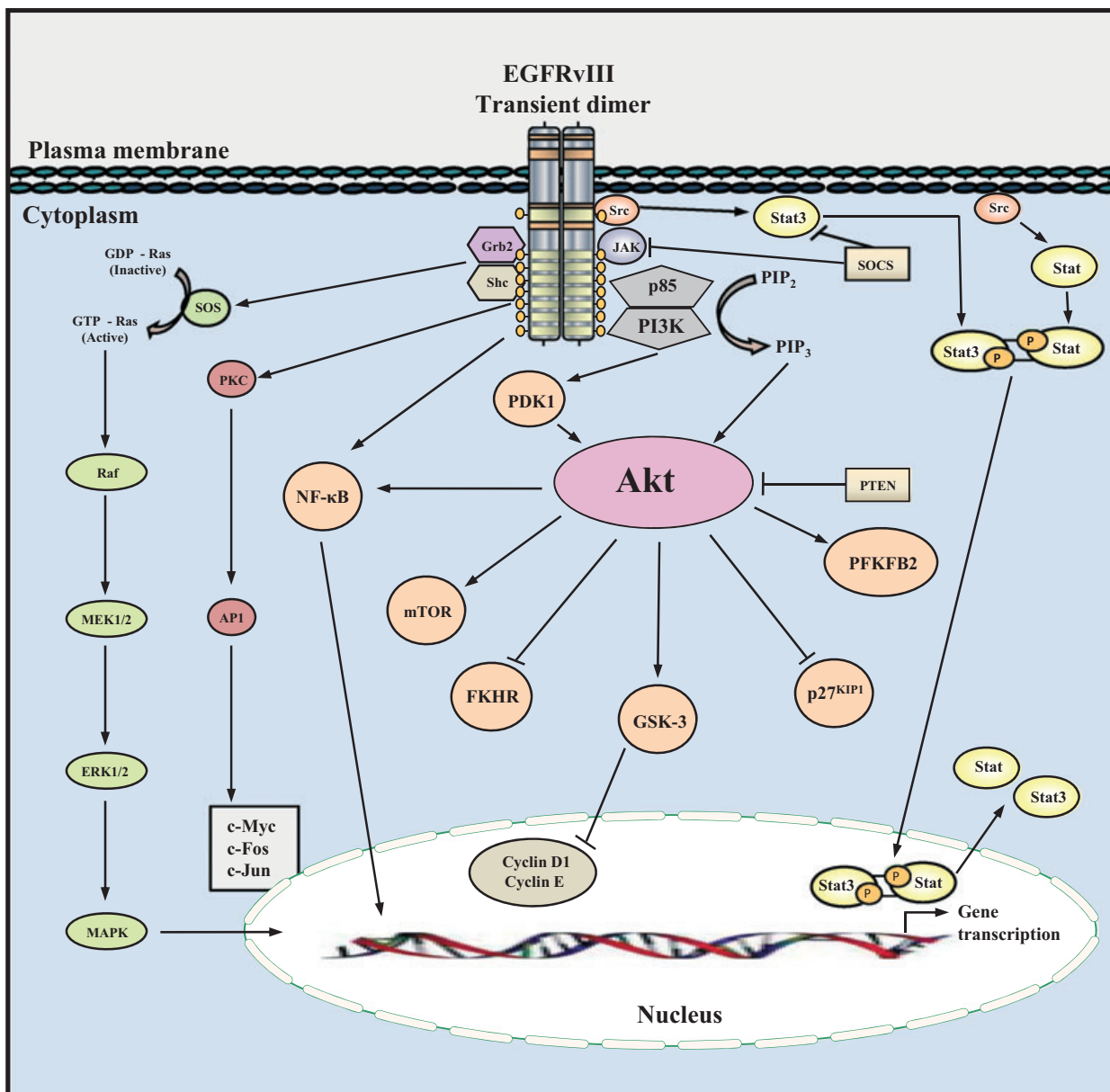


Fig. 2. Downstream signaling pathways of EGFRvIII. Multiple downstream signaling pathways are initiated by EGFRvIII. The phosphatidylinositol 3-kinase (PI3K)/Akt pathway has been identified as the signaling cascade that is preferentially activated by EGFRvIII, particularly in glioblastoma multiforme (GBM).

conferred by EGFRvIII seems to be more prominent *in vivo* than *in vitro*. For example, transfection with EGFRvIII had only a modest effect on the *in vitro* growth characteristics of the GBM cell line U87MG, even under low serum conditions, but these cells had a marked growth advantage when grown as tumor xenografts in nude mice [4,146,183]. Furthermore, the growth advantage conferred by EGFRvIII *in vivo* resulted in the selective clonal expansion of EGFRvIII-expressing cells *in vivo* [146]. This was dem-

onstrated by mixing U87MG.Δ2-7 cells (U87MG parental cells which have been transfected to overexpress EGFRvIII) with parental U87MG cells at a ratio of 1 : 10 000 and subsequently implanting them into the brains of nude mice. At 4–5 weeks post inoculation, this cell ratio was 20 : 1 [146].

The pro-tumorigenic effects of EGFRvIII seem to rely directly on its ability to signal [17,146,188], because transfection of a kinase-dead version of EGFRvIII into U87MG cells did not enhance

xenograft growth. Furthermore, all five major auto-phosphorylation sites in the carboxy-terminal region of EGFRvIII are required for effective signaling, because even single point mutations at these sites, particularly of the tyrosine residues Tyr1068, Tyr1148 and Tyr1173, were sufficient to abolish most of the *in vivo* growth advantage conferred by EGFRvIII [17]. Lastly, although *EGFR* gene amplification is nearly always found together with EGFRvIII expression, wt EGFR expression is not required for the pro-tumorigenic effects of EGFRvIII as these effects have been shown even in the absence of wt EGFR expression [52].

The strength of the constitutive signaling mediated by EGFRvIII on the cell surface is a matter of debate, but many studies suggest that it is approximately 10% of the intensity of ligand-induced wt EGFR signaling [10,16,17,123]. EGFRvIII signaling, however, is significantly enhanced by the prolonged retention of this receptor on the cell surface because of its impaired endocytosis [17,193]. Paradoxically, it is the low level of EGFRvIII signaling, especially the hypo-phosphorylation at Tyr1045, that prevents the receptor from engaging with the endocytic proteins Cbl, CIN85 and endophilin and leads to its retention on the cell surface [68]. Pharmacological attenuation of wt EGFR signaling to approximately 20% of normal activity levels results in impaired interaction with the Cbl–CIN85–endophilin complex, as well as the absence of polyubiquitination and reduced internalization of the receptor; these observations are consistent with EGFRvIII downregulation. The importance of impaired downregulation has been further confirmed by Davies *et al.* [67], who showed that overexpression of the Cbl family of ubiquitin ligases made it possible to induce the polyubiquitination and degradation of EGFRvIII with a subsequent loss of tumorigenicity.

EGFRvIII activates several downstream pathways (Fig. 2), but a considerable amount of evidence indicates that it preferentially activates the PI3K/Akt signal transduction pathway [7,51,179–181]. EGFRvIII expression is tightly correlated with the activation of downstream targets of PI3K/Akt, including the mammalian target of rapamycin (mTOR), the fork-head (FOX) transcription factor family and S6 [182]. Recently, EGFRvIII has been shown to activate mTORC2 (CREB-regulated transcription co-activator 2) via the PI3K/Akt pathway, which in turn leads to stimulation of the NF- κ B pathway and resistance to chemotherapy [194]. Another consequence of EGFRvIII activation of PI3K/Akt is increased proliferation and cell cycle progression mediated by a decrease in the level of p27^{KIP1}, a cyclin-dependent

kinase (CDK) inhibitor that binds and inactivates CDK2–cyclin E complexes and thereby inhibits the transition of cells from the G1 phase to the S phase [173]. The significance of PI3K/Akt activation by EGFRvIII has been confirmed by Klingler-Hoffmann *et al.* [178], who showed that treatment of U87MG. Δ 2-7 cells with the PI3K inhibitor wortmannin, or by reconstitution of the physiological levels of PTEN (a negative regulator of PI3K), resulted in the ablation of the EGFRvIII-conferred growth advantage. Narita *et al.* [173] also demonstrated that overexpression of dominant-negative Akt in U87MG. Δ 2-7 cells restored p27^{KIP1} to levels similar to those seen in parental U87MG cells, causing G1 cell cycle arrest and the loss of tumorigenicity. Selective activation of the PI3K/Akt pathway by EGFRvIII is thought to mediate the resistance to radiation that is observed in EGFRvIII-positive GBM [21,50,182,195–197]. EGFRvIII signaling via the PI3K/Akt pathway may be facilitated by associated loss or mutation of the *PTEN* gene, which occurs in approximately 40% of GBM samples that express EGFRvIII [153,174–176].

Several studies have shown that EGFRvIII and Src family kinases (SFKs) work cooperatively to enhance GBM tumorigenicity [103,198]. The expression of EGFRvIII caused activating phosphorylation of the receptor and physical association of the SFK members Src and Fyn, which in turn promoted tumor growth and motility [103]. Genetic or pharmacological inhibition of SFKs inhibited cell motility *in vitro* and growth of EGFRvIII-expressing GBM xenografts *in vivo* [103,199]. More recently, it was shown that the phosphorylation of Tyr772 on SFKs by EGFRvIII leads to the phosphorylation of dedicator of cytokinesis 1 (DOCK1; also known as DOCK180), a guanine nucleotide exchange factor with roles in cell motility, survival and proliferation [199]. Genetic ablation of *DOCK180* blocked the EGFRvIII-mediated tumorigenicity of GBM cells [199].

The enhanced tumorigenicity of GBM cells expressing EGFRvIII is also associated with enhanced angiogenesis. Wu *et al.* [184] showed that the increased tumorigenicity conferred by transfection of GBM cell lines with EGFRvIII could be reversed by genetically inhibiting the NF- κ B pathway. This reversal was associated with concurrent reductions in vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8) expression and with reductions in tumor angiogenesis. These studies were confirmed and extended by Bonavia *et al.* [200], who showed that EGFRvIII transfection increased IL-8 expression in GBM cells by 60-fold through the NF- κ B pathway. RNA-interference-mediated knockdown of the IL-8 pathway or the NF- κ B

pathway inhibited GBM xenograft growth and attenuated angiogenesis.

EGFRvIII dimerization

Most publications indicate that EGFRvIII predominantly forms transient homodimers [17,52,201]. The findings of an early publication, showing constitutive dimerization of EGFRvIII, have not been reproduced [16], and another study reporting significant dimerization used non-physiological conditions that included an extensive and lengthy purification [29]. As autophosphorylation of the EGFR only occurs in *trans* after the formation of an asymmetric dimer [202], transient EGFRvIII homodimers must exist. By using differentially tagged EGFRvIII molecules, we were able to demonstrate this transient interaction [52]. Furthermore, we have also shown that EGFRvIII can interact transiently with wt EGFR [52]. Recently, we demonstrated that the transient EGFRvIII homodimer is stabilized by disulfide bonds through the free amino-terminal cysteines that are created by the deletion event [203]. Finally, forced dimerization of EGFRvIII by using genetic strategies enhances receptor phosphorylation and downstream signaling, leading to increased GBM cell proliferation [204]. Taken together, these data confirm the critical role of EGFRvIII dimerization for signaling.

EGFRvIII activates multiple receptors through differing mechanisms

The direct interplay between EGFRvIII and wt EGFR has not been examined extensively. Co-expression of the two receptors in IL-3 dependent BaF/3 caused increased survival and proliferation in the absence of IL-3 compared with expression of EGFRvIII alone; wt EGFR expressed alone has no activity in these cells in the absence of ligand [52]. When EGFRvIII is expressed in U251-MG glioma cells it induces expression of the EGFR ligands TGF- α and HB-EGF [70]. Furthermore, neutralization of HB-EGF with antibodies reduced U251-MG cell proliferation suggesting that this EGFRvIII induced autocrine loop has a role in EGFRvIII-mediated tumorigenicity.

White and colleagues [205] conducted a mass spectral analysis of the phospho-proteome activated by EGFRvIII in U87MG cells. Along with numerous intracellular targets phosphorylated by EGFRvIII, the RTK c-Met also appeared to be phosphorylated in response to EGFRvIII expression. Additional studies have confirmed that c-Met is activated by EGFRvIII and have shown that this occurs independently of

hepatocyte growth factor (HGF), the c-Met ligand [205]. Recently, it was shown that the activation of c-Met by EGFRvIII is a major mechanism underlying the previously reported activation of the transcription factor Stat3 [206].

Using phosphorylated RTK arrays, we established that EGFRvIII activates not only c-Met in the U87MG cell line but many other RTKs, including platelet-derived growth factor receptor β (PDGFR β) and VEGF receptor 2 (VEGFR2) [106]. Using panitumumab, an EGFR-specific antibody that inhibits EGFRvIII activation and phosphorylation, we were able to block the phosphorylation of c-Met and PDGFR β [106]. This verified that the activation of RTKs was mediated by EGFRvIII and was not an artifact of clonal selection following transfection. The ligand-independent activation of c-Met was confirmed by using neutralizing antibodies specific for HGF. Although the evidence suggests that EGFRvIII mediates the activation of c-Met through a direct interaction, the possibility of a downstream mechanism cannot be completely dismissed. Notably, these observations help explain the potent tumorigenicity of EGFRvIII despite its low level of activation.

EGFRvIII has been shown to stimulate the production of cytokines, including IL-6 and leukemia inhibitory factor (LIF), both of which signal through the gp130 complex [207]. Importantly, these cytokines have been shown to activate overexpressed wt EGFR in neighboring GBM cells through a mechanism involving crosstalk between gp130 and wt EGFR, and activation of wt EGFR by this mechanism led to enhanced GBM cell proliferation [208]. Thus, EGFRvIII contributes to the growth of surrounding GBM cells through this field effect. More generally, this indicates that EGFRvIII actively contributes to the heterogeneity of GBM by acting indirectly on neighboring cells that are EGFRvIII negative. This hypothesis is entirely consistent with the observation that wt *EGFR* amplification and EGFRvIII expression are usually observed together. It may also explain why, in patients, the pronounced growth advantage mediated by EGFRvIII does not lead to a homogeneous population of cells all of which express the EGFRvIII receptor.

Role of EGFRvIII in metabolism

The PI3K/Akt/mTOR pathway, which is preferentially activated by EGFRvIII [7,51,179–181], is associated with changes in cell metabolism, including increased anaerobic glycolysis and lipogenesis [97,209,210]; these processes are important for continuous tumor cell

proliferation [209,210]. AMP-activated protein kinase (AMPK), an enzyme that plays a key role in regulating cellular metabolic processes including lipogenesis, inhibits mTOR signaling when activated. Using the AMPK agonist AICAR, which reportedly mediates its anti-tumor effects through forced AMPK activation and subsequent mTOR inhibition [211,212], Guo *et al.* [97] examined the link between EGFRvIII, the PI3K/Akt/mTOR pathway (Fig. 2) and lipogenesis. Expression of EGFRvIII in U87MG cells caused an increase in intracellular fatty acid production *in vitro* and enhanced ^{18}F -fluorodeoxyglucose (FDG) uptake *in vivo* [97], suggesting a role for EGFRvIII in lipogenesis and cell metabolism. Treatment with AICAR was found to significantly decrease ^{18}F -FDG uptake *in vivo* and intracellular fatty acid production *in vitro* [97]. Collectively, these results suggest that activation of the PI3K/Akt/mTOR pathway by EGFRvIII promotes tumor growth through coordinated regulation of cell metabolism and lipogenesis. Finally, it has been shown that SFK activation of EGFRvIII stimulates the translocation of EGFRvIII to the mitochondria, where it enhances survival in low glucose conditions by an unknown mechanism [198].

Targeting EGFRvIII as a therapeutic modality

Whilst it is beyond the scope of this review to comprehensively discuss the therapeutic value of inhibiting EGFRvIII, a brief summary is useful to emphasize the importance of EGFRvIII as a clinical target. Pre-clinical *in vivo* studies consistently show that targeting of EGFRvIII can be therapeutically useful in brain tumors [32,62,73,95,118,213,214]. However, compared with wt EGFR, EGFRvIII appears to be relatively resistant to treatment with conventional anti-EGFR agents such as ligand blocking monoclonal antibodies [72,76,85,215–218] or EGFR tyrosine kinase inhibitors [37,50,132,219]. A number of alternative approaches have shown clinical promise and are in clinical trials, notably mAb806 and rindopepimut (CDX-110). mAb806 is a novel anti-EGFR antibody which was generated by vaccinating mice with EGFR-negative NR6 fibroblast cells transfected with EGFRvIII [5]. mAb806 targets a conformationally exposed epitope on wt EGFR when it is overexpressed in tumor cells or in the presence of oncogenic mutations such as EGFRvIII [220]. Not only is mAb806 less toxic than other anti-EGFR antibodies [221], it shows excellent blood–brain barrier penetration and localization to *in situ* GBM in patients [222]. mAb806, currently named ABT-806, has just completed phase 1 testing

(trial identifiers NCT01255657, NCT01472003 and NCT01406119). A trial of ABT-414, an antibody–drug conjugate based on mAb806, is currently recruiting patients with newly diagnosed or recurrent GBM for treatment with concurrent radiotherapy and/or temozolomide (trial identifier NCT01800695). Rindopepimut is a 14-amino-acid peptide bridging the unique glycine at the site of the EGFRvIII mutation that is conjugated with keyhole limpet hemocyanin [223]. Phase 2 data combining rindopepimut vaccination with first-line chemo-radiation for newly diagnosed GBM were encouraging compared with historical data and treatment was well tolerated [223]. A phase 3 study of rindopepimut in the same setting is currently under way (trial identifier NCT01480479) as is a study of rindopepimut in patients with relapsed GBM in combination with bevacizumab, a monoclonal antibody against VEGF, or as a single agent in those who have failed bevacizumab (trial identifier NCT01498328). The role of anti-EGFRvIII therapy in other tumor types is still to be addressed.

Conclusion

EGFRvIII is expressed at a relatively high frequency in GBM tumors but determining its frequency and significance in other tumor types will require additional studies. Recent work has begun to unravel the signaling networks that are used by EGFRvIII. Clearly, there is overlap with the pathways that are activated by wt EGFR; however, significant differences are starting to emerge, especially with respect to the co-activation of other cell-surface receptors by EGFRvIII. So far, these co-activation experiments have been performed in a limited range of transfected GBM cell lines and need to be reproducible in EGFRvIII-expressing xenografts, GBM spheroid lines or other cell lines that naturally express EGFRvIII. The assortment of RTKs that can be activated by EGFRvIII remains unknown; it is unclear whether EGFRvIII can activate any RTK that is expressed on the cell surface at a significant level or whether it only activates a specific subset. Although further insight into the tumor-promoting activities of EGFRvIII will improve our understanding of GBM development and progression, it is the broader implications – such as the maintenance of tumor heterogeneity, the rewiring of signaling pathways, the co-opting of other receptors and the metabolism of cancer cells – that make understanding this receptor so important. Lastly, the therapeutic potential of targeting EGFRvIII in brain and other tumors is becoming increasingly apparent, with agents against EGFRvIII now in clinical trials.

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