Review article

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Epidermal growth factor receptor in glioblastoma

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Abstract

We compiled the current state of knowledge about the epidermal growth factor receptor (EGFR) in glioblastoma. Glioblastoma is one of the most common primary brain tumours and has an unfavourable prognosis despite aggressive treatment. These factors stimulate new research trials and a recent area of interest of neurooncologists is EGFR. This molecule is frequently altered in glioblastoma and constitutes the potential target for therapy. We decided to review the literature on biological structure of that molecule, its biological activity and the role in GBL with potential targeting it in the future neurooncological practice.

Key words: *glioblastoma, epidermal growth factor receptor, MAPK, EGFRvIII, pathology, genetics*

Introduction

Astrocytic tumours of the central nervous system (CNS) are the most common neoplasms of the brain. These tumours form two well-defined groups of lesions: well circumscribed astrocytomas and diffusely infiltrating ones. The former are composed of several entities, such as pilocytic astrocytoma, subependymal giant cell astrocytoma and pleomorphic xanthoastrocytoma. The latter are divided into diffuse astrocytomas, WHO Grade II; anaplastic astrocytoma, WHO Grade III and glioblastoma (GBM), WHO Grade IV [1]. Glioblastoma is one of the most common brain tumours and accounts for 12-15% intracranial neoplasms [2]. High frequency of glioblastoma and its grim prognosis despite aggressive treatment stimulates new research trials. A recent area of interest is the epidermal growth factor receptor (EGFR), since its abnormalities are one of the most common molecular aberrations in glioblastoma. We decided to compile the current state of knowledge about that molecule, its biological activity and the role in GBL with potential targeting it in the future neurooncological practice.

Molecular structure of the EGF receptor

The epidermal growth factor receptor (EGFR) belongs to a family of four closely related receptors that includes also HER-2/neu (ErbB-2), HER-3 (ErbB-3), and HER-4 (ErbB-4) [3,4]. They are membrane-bound receptors that form type I receptor tyrosine kinase family, and the organization of other receptor kinase families (type II – insulin receptor; type III – *c-kit, c-fms*) has already been defined. The EGFR has close homology to the transforming gene of the avian erythroblastosis virus (v-erbB). This suggested that the v-erbB gene is the oncogenic version of the *EGFR*.

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Therefore, ErbB1 is interchangeably used as a synonym for the *EGFR* [5].

The epidermal growth factor receptor was originally cloned in 1984 [6]. It has a molecular mass of 170 kDa [7], and consists of 28 exons [8]. The *EGFR* is localized to chromosome 7p11-13 [9-11] and its protein is synthesized from a 1210-residue polypeptide precursor, as a result of N-terminal sequence cleavage. The final product of that cleavage is the 1186-residue protein, which functionally resides within the cell membrane [12]. This glycoprotein consists of three portions: extracellular, transmembrane and intracellular (Fig. 1). The *extracellular portion* consists of four domains: I (amino acids 1-165); II (a.k.a. CR1; amino acids 166-309); III (amino acids 310-481); and IV (a.k.a. CR2; amino acids 482-621). Domains I and III have 37% sequence homology, are cysteine-poor and contain the site for ligand binding. Cysteine-rich domains II and IV contain N-linked glycosylation sites and disulfide bonds that determine the tertiary conformation of the external portion of the molecule [13,14]. Domains I, II, and III of the EGFR have β-helix tertiary configuration with structural and sequence homology to the first three domains of the type I insulin-like growth factor receptor [15]. An EGFR ligand binds directly to domain III [16]. The II and IV domains consist of a number of small modules, each appearing to be held together by one or two disulfide bonds. A large loop that protrudes from the back of the II domain makes a molecular contact with the respective domain of the other receptor. Dimer formation between two EGFR molecules takes place on a ligand binding and results in kinase activation [16,17].

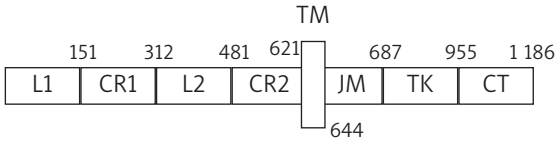

Fig. 1. Schematic representation of domains of the epidermal growth factor receptor sequence (according to [17]). The abbreviations used: L and CR, for the ligand binding and the cysteine-rich domains, respectively (a.k.a. I (L1), II (CR1), III (L2), and IV (CR2) or S1 (CR1) and S2 (CR1), where L and S refer to large and small); JM, juxtamembrane domain; CT, carboxy-terminal phosphorylation site; TK, tyrosine kinase domain. The transmembrane domain (residues 622–644, TM) is between the CR2 and the juxtamembrane domain

The identification that the *transmembrane domain* consists of residues 622-644 was performed by visual analysis of the EGFR sequence [12]. The nuclear magnetic resonance analysis of a peptide corresponding to the EGFR transmembrane domain and to the beginning of the cytoplasmic domain indicates that residues 626–647 are α-helical. An *intracellular domain* contains an uninterrupted tyrosine kinase site and multiple autophosphorylation sites clustered at the C-terminal tail. The carboxy-terminal domain of the EGFR contains tyrosine residues that may be phosphorylated and then they modulate EGFR-mediated signal transduction. There are also several serine/threonine residues (and another tyrosine residue) where phosphorylation has been inferred to be important for the receptor downregulation processes and sequences thought to be necessary for endocytosis. The juxtamembrane region appears to initiate a number of different cascades of reactions that ultimately result in DNA replication and cell division [3,17]. The earliest consequence of kinase activation is autophosphorylation of its own residues. This is followed by phosphorylation and activation of signal transducers, which lead to mitogenesis.

Signalling pathways of the EGFR

There are several ligands which bind with high affinity to the EGFR. The first known ligand is the epidermal growth factor (EGF), which is a small polypeptide of 53 amino acids derived by proteolytic processing from a large protein precursor molecule of 1168 amino acids [18]. The second ligand identified was the transforming growth factor-alpha (TGFα), which has 50 amino acids and is derived from a precursor molecule containing 160 amino acids [19]. Other ligands, such as TDGF-1, amphiregulin, betacellulin, heparin-binding EGF and epiregulin, are also derived from larger peptide precursors [20]. They were identified as possible additional ligands of the EGFR due to structural homologies (reviewed by Novak et al. [21]).

Intracellular signal tranduction from activated EGFR

The EGFR is important for the maintenance of the normal cellular function and survival. In neoplastic

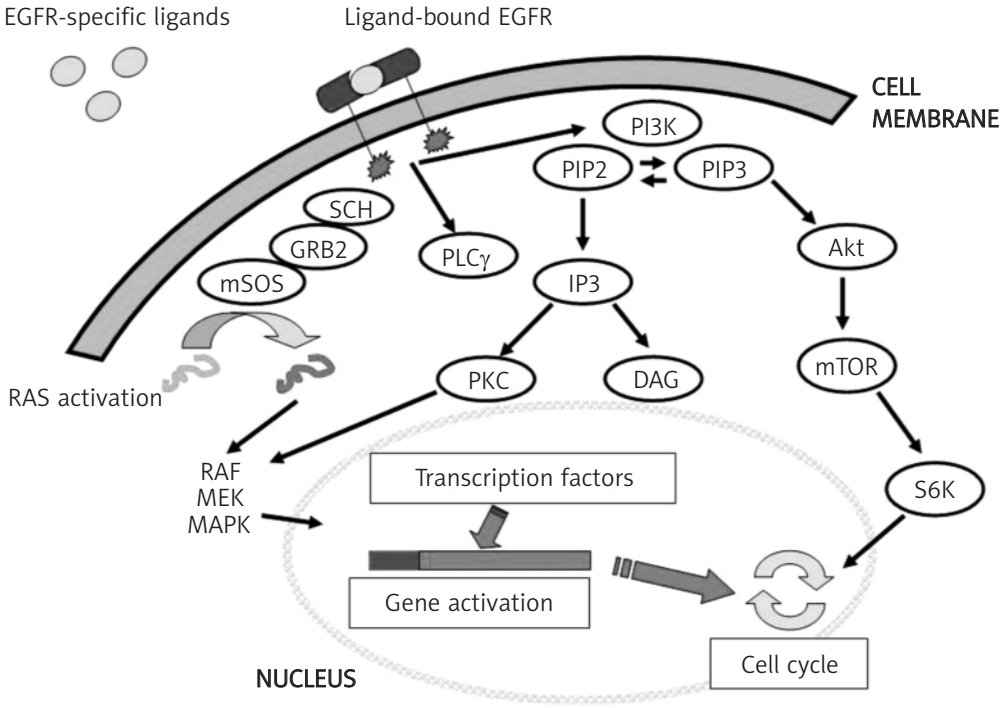

Fig. 2. Intracellular signalling pathways of the EGFR. Abbreviations: GRB2 – growth factor receptor-bound protein-2, mSOS – son of sevenless, PI3K – phosphatidylinositol 3-kinase, PIP2 – phosphatidylinositol-2, PIP3 – phosphatidylinositol-3, S6K – p70 S6 kinase, mTOR – mammalian target of rifampicin, PKC – protein kinase C, PLCγ – phospolipase Cγ, IP3 – inositol 1,4,5-triphosphate, DAG – 1,3-diacylglycerol, MAPK – mitogen-activated protein kinase

cells it contributes to their growth and survival through various divergent pathways. Dimerization of ligand-bound EGFR results in receptor autophosporylation. In this process one receptor molecule phosphorylates the other in the dimer [22]. The signal is then propagated by the cascade activation of several intracellular transducers. The main kinases involved in this process are mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K). Indirectly, they induce cell proliferation, tumour invasion and angiogenesis by subsequent phosphorylation of several transducers. These separate steps are shown in Fig. 2.

Ligand binding by EGFR results in activation of the adapter proteins, such as mSOS and GRB2. This pathway ultimately leads to phosphorylation of ras protein. Ras, a GTPase, may present in the active (GTP binding) and inactive (GDP binding) forms. Inactivation of ras is attained by dephosphorylation of GTP-binding form by GAP (GTPase activating protein). The active form of ras transfers the signal down to the distal kinases like MAP kinase, MEK or RAF.

The other pathway leads through the activation of PI3K that transduces the signal from the receptor by generating the lipid second messenger, phosphatidylinositol-3,4,5 triphosphate (PIP3). It is derived from phosphatidylinositol-3,4 biphosphate (PIP2) by phosphorylation at the 3`-OH position of the inositol ring [23]. This reaction is opposed by a dual-specific phosphatase, PTEN, which dephosphorylates PIP2 and PIP3 [23]. In addition, PI3K phosphorylates multiple cellular proteins, including serine/threonine family of kinases, Akt. It consists of three members – Akt1/PKBα, Akt2/PKBβ and Akt3/PKBγ which share a high degree of structural similarity [24,25]. These very important molecules promoting many pro-tumorigenic responses regulate the activity of p70 S6 kinase through mTOR. The Akt targets or substrates play a key role in regulating

critical cellular functions including proliferation, apoptosis, glucose homeostasis, cell size, nutrient response and DNA damage [26].

Overexpression and amplification of the EGFR in glioblastoma

The *EGFR* is associated with the growth of malignant cells. Whereas its expression in the normal cells is estimated as around 40.000-100.000 receptors per cell [27], in the malignant tumours it may reach the level of 2 million per cell [28].

The *EGFR* amplification and mRNA overexpression are frequent in high grade gliomas of astrocytic origin, and are always strongly associated with an increased level of the EGFR protein [29,30]. Protein overexpression without gene amplification has been reported in up to 27% of GBMs [31], but less malignant astrocytomas and oligodendrogliomas were also reported to demonstrate the EGFR overexpression without the underlying gene amplification [32]. The true molecular background of that phenomenon is unclear at the moment, but enhanced EGFR transcription by some transcription factors (ETF, SP1, TP53) or cytokines (TGFβ1, interferon-γ) have been suggested.

The *EGFR* amplification is generally associated with high protein expression levels, as measured by

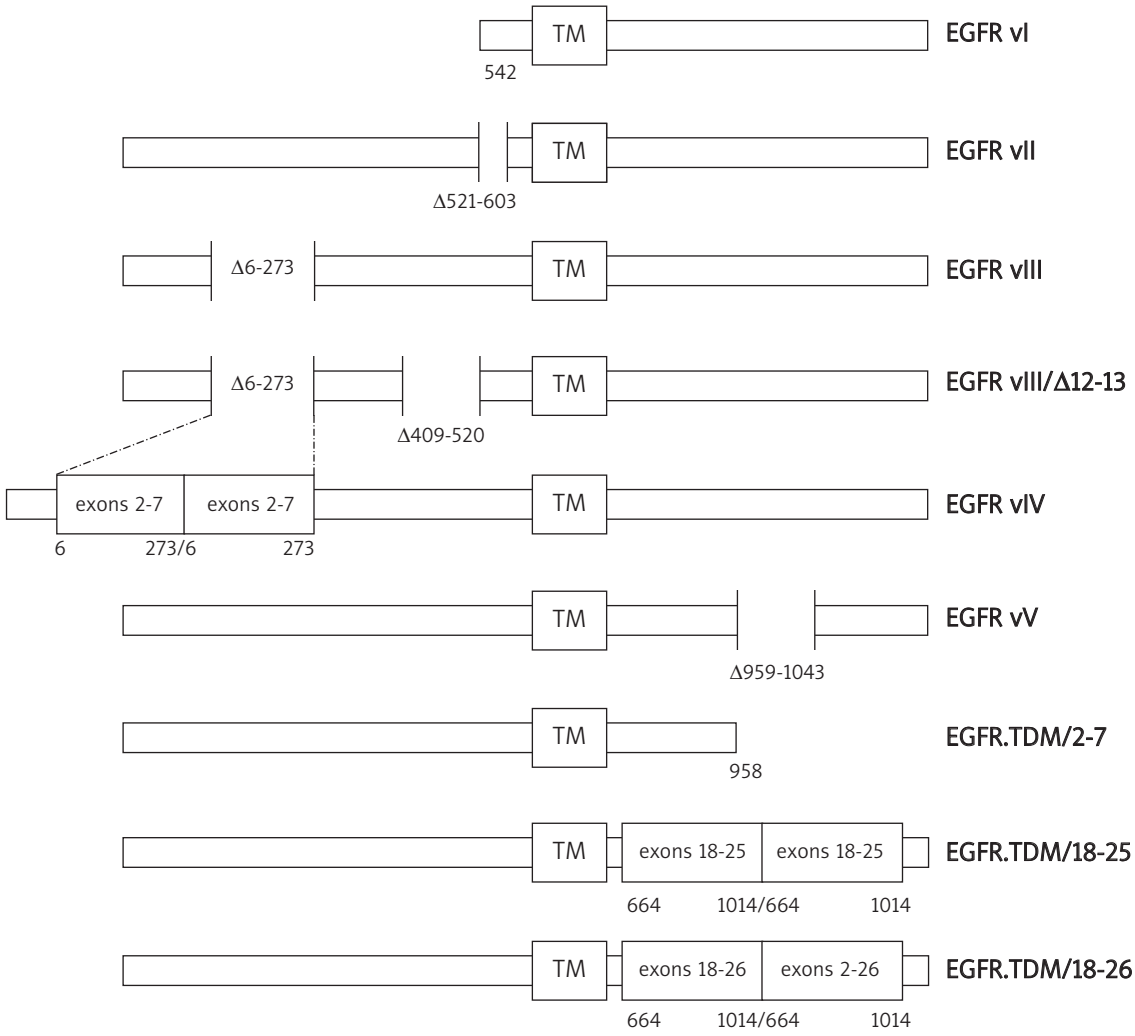

Fig. 3. Mutations of the EGFR (reviewed by Kuan et al. [89])

Western analysis [33,34]. Observation of the frequent amplification of the *EGFR* in GBM was initially reported in 1985 by Libermann et al. [35], and this association has been confirmed in several subsequent studies. Amplification of the *EGFR* has been described in about 30-62% of GBM [30,34-43], but it was infrequent in anaplastic astrocytomas, reported to occur in 3% of cases [11,44].

Clinically-based separation of secondary GBMs, which develop as a result of progression from a pre-existent lower grade astrocytoma, and primary GBM, developing in a short time without precursor lesion, was validated by molecular findings. The EGFR amplification appeared to be prevalent in primary GBM (~40% of cases) [31,45], but it was not found in secondary GBM [31]. This difference was obvious also at the protein level, as immunohistochemical analysis of primary and secondary GBMs showed the EGFR overexpression in 60% and 10% of cases, respectively.

Several studies have found that the EGFR overexpression in GBM varied with age of the patient. The EGFR amplification/overexpression was significantly more frequent in GBM in patients older than 55 years of age [46-50] and this reflects also the age prevalence of primary glioblastomas.

Few investigators examined the EGFR overexpression in pediatric GBMs. The results were similar to those obtained in studies on young adult patients with GBM. None of 18 pediatric GBMs had the EGFR amplification in a study by Kraus et al. [51]. In another study, it was found in only 2 of 13 (17%) cases of pediatric primary GBMs [47].

Genomic variants of the EGFR

In early studies on the EGFR amplification several groups identified simultaneous structural abnormalities of the amplified receptor [35,52,53]. Several genomic variants of the EGFR have been detected, each of them showing identical splicing sites within each group (see Fig. 3). These mutants arise in a process of internal deletions or sequence duplication and are not expressed in the normal tissue [53-56]. Most of the identified mutants (67%) contain an identical deletion of part of the extracellular domain of the EGFR molecule [54]. This mutant was called EGFRvIII (a.k.a EGFR Δ2–7) [57]. It has an in-frame deletion of 801 base pairs, corresponding to exons 2-7 in the mRNA. Loss of this portion of the gene is suggested to be a consequence of recombination of highly repetitive sequences (Alu) within the introns 1 and 7 [58]. At the protein level, this results in the deletion of amino acids 6-273 in the extracellular domain and the generation of a glycine at the fusion site [59]. This truncated mutant receptor has a molecular mass of 145 kDa compared with that of 170 kDa for wild type EGFR (EGFRwt).

Confocal microscopy analysis confirmed that subcellular localization of EGFRvIII was identical to that described for EGFRwt. Both receptors had predominant cell membrane expression, but they were also identified in the perinuclear area, suggestive of localization to the Golgi region [60]. Neither EGFRwt nor EGFRvIII was found within the nucleus [60]. This subcellular distribution of the receptors provides an excellent opportunity for use of target-aimed treatment specific for EGFRvIII (see below).

There are some functional differences between the EGFRvIII and EGFRwt. The activity of EGFRvIII is not influenced by EGF or TGFα, as the mutation results in the loss of ligand binding site of the receptor. However, EGFRvIII has constitutively active tyrosine kinase domain and has a defective downregulation activity [61]. A weak but constitutive activity of the truncated receptor results in enhanced tumorigenicity in nude mice [62]. The functional background of that phenomenon depends on increased proliferation and decrease in apoptosis of tumour cells bearing EGFRvIII. Furthermore, overexpression of EGFRwt did not confer a similar growth advantage [61; 63]. The molecular mechanism by which the EGFRvIII acquires transforming activity is not yet clear. The EGFRvIII has been found to be constitutively associated with signalling adapter proteins Shc and Grb2, similarly to EGFRwt (see above). These molecules are involved in the recruitment of Ras to activated receptors, and that process is not dependent on receptor dimerization [64-66]. Studies of Fernandes et al. [67] showed that the high kinase activity of the EGFRvIII is due to self-dimerization, and that the kinase activity of the dimeric EGFRvIII molecule is comparable to that of the EGF-stimulated wild-type receptor. The patterns of phosphorylation of both the EGFRwt and EGFRvIII receptors are similar, and the receptor–receptor self-association is highly dependent on a conformation induced by N-linked core glycosylation [67].

Feldkamp et al. [10] confirmed that constitutively active EGFRvIII enhanced the growth of glioblastoma cells through the same signalling pathway of Ras-GTP as EGFRwt. Moscatello et al. [68] demonstrated that

EGFRvIII-positive cells demonstrate high levels of PI3K activity which resulted from the kinase activity of the receptor. Therefore, PI3K may play an essential role in EGFRvIII transformation of the cells. In addition, EGFRvIII up-regulates expression of vascular endothelial growth factor (VEGF) in glioblastoma by activation of PI3K-dependent signalling pathway [69]. Likewise, the c-Jun N-terminal kinase (JNK) pathway was found to be constitutively active in the EGFRvIII-positive cells [70] and high JNK activity was not found in the cells overexpressing the EGFRwt. This implicates that JNK pathway plays an important role in cell transformation by EGFRvIII and is highly specific for this variant receptor.

The EGFRvIII was found in about 32-41% glioblastomas with EGFRwt overexpression [39,54,71,72], and 27-43% of all GBMs [39,71]. The distribution and strength of EGFRvIII expression may vary. In some cases it is more abundant in the perivascular regions and less intense or lacking in the perinecrotic areas [39,72]. In the recent study, we have shown that the tumour cells usually demonstrate overexpression of both EGFRwt and EGFRvIII, however, in some cases mutated EGFR is less extensively present in the tumour bulk [39]. Likewise, molecular quantitative real time PCR showed that *EGFRvIII* was predominantly amplified in only three of eight cases having amplification of both wild type and mutated EGF receptor genes [39]. These two factors may, thus, influence the therapeutical success of specifically EGFRvIII-aimed therapy.

Correlation between the EGFR status and other molecular markers

The relationship of the mutation status of *TP53* and *EGFR* amplification in glioblastomas has been the subject of several investigations. In general, the association between the *TP53* and *EGFR* status showed a tendency for under-representation of combination of *TP53* mutation and the *EGFR* amplification in series GBMs, but this was not statistically significant [73]. The *TP53* mutation and EGFR amplification were negatively associated in another study of 123 GBM cases [45,74].

The *PTEN* suppressor gene, which encodes dual-specificity phosphatase that negatively regulates molecular pathways used by the EGFR proteins family, is located on chromosome 10q23 [75,76]. Despite frequent association between the *EGFR* amplification and loss of 10q in glioblastomas [43], no significant correlation was found between the *EGFR* amplification and *PTEN* mutations [31,77].

In glioblastomas, the *EGFR* amplification and *CDKN2A/p16* deletion are frequently simultaneous molecular alterations [74,78]. In contrast, the *TP53* mutation and *EGFR* amplification were mutually exclusive in GBL and they were considered genetic hallmarks of secondary and primary glioblastomas, respectively [79,80].

The EGFR amplification and/or overexpression status and prognosis and survival in patients with brain tumours

The prognostic implications of the EGFR amplification/overexpression in brain tumours are controversial. Some authors did not find any influence of the EGFR amplification/overexpression on survival of the patients [37,81,82] while the others concluded that these alterations were a negative prognostic factor [34,50,83,84].

The EGFR expression showed a significant association with the prognosis in GBM patients' subset, defined by age and p53 status. This relationship was identified through the unexpected finding that the EGFR positivity by immunohistochemistry was related to improved survival only in elder patients. A shorter survival was observed in younger patients with EGFR positivity. Moreover, among the younger patients, the EGFR predicted worse prognosis only in those with tumours that were p53-negative by immunohistochemistry [46].

The finding that the *EGFR* amplification is a predictor of longer survival only in older GBM patients was confirmed by Smith et al [85].

In contrast with the above studies, a large population-based study found no association of the *EGFR* amplification and survival in GBM patients at any age. This study demonstrated an impressive association of patients' age and the EGFR amplification, i.e. the EGFR amplification was not detected in any GBMs of patients below 35 years of age. Such age-related distribution of the EGFR parallels the age distribution of primary GBMs [48].

A recent study has demonstrated that patients with ΔEGFR-positive GBM have shorter life expectancies [86], suggesting that this specific-specific genetic alteration may be related to higher aggressiveness of GBMs.

Prospects of using the EGFR-targeted therapy in glioblastoma

GBM is a primary high grade astrocytic neoplasm which remains one of the most lethal malignancies, despite a considerable progress in radiation and chemotherapy. From the reviewed results of the recent studies of the EGFR, it is apparent that this molecule plays a pivotal role in the tumorigenesis and anti-EGFR targeted therapy may appear a promising tool against GBL, most likely in conjunction with other treatment modalities.

There exist a few treatment approaches to the EGFR molecule on the cancer cell. The most extensively studied are: specific antibody therapy by means of unarmed antibodies or antibodies conjugated with toxins, liposomes or nuclides, and the use of inhibitors of the receptor tyrosine kinase. There are several types of monoclonal antibodies directed against the EGFRwt. Their use results in blocking access to the receptor for its ligands (cetuximab) and/or rapid internalisation of the receptor (ABX-EGF) [87]. As the EGFRwt occurs also on the surface of normal cells, side effects may limit its use.

The mutated form of EGFR, i.e. EGFRvIII, provides an excellent target for treatment, as it occurs exclusively on the specific cell surface and this decreases the undesirable effects of treatment that are met with anti-EGFRwt antibodies. Thus, our understanding of the distribution of the wild type and various mutated forms of the EGFR in human gliomas is critical for development and implementation of anti-EGFR medications targeting specific form of receptor expressed by specific cells [39].

EGFR inhibitors are molecules that specifically inactivate the receptor tyrosine kinase. These are mostly derivatives of anilinoquinazoline that competitively bind to the kinase domain with ATP. They will most likely be used together with chemotherapeutic agents, as part of complex treatment protocols. The extensive review of the latest achievements in targeted therapy of GBL can be found in the recent review by Mischel and Cloughesy [88].

Further investigations of intracellular interactions of the EGFR and a neoplastic cell are necessary to establish the most effective treatment regimens in glioblastoma. Development of new and successful treatment strategies will depend largely on our understanding of basic pathogenetic processes underlying its development.

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