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Nuclear Signaling of EGFR and EGFRvIII in Glioblastoma

Anupama E. Gururaj, Oliver Bögler and Khatri Latha
Department of Neurosurgery, University of Texas M. D. Anderson Cancer Center, Houston, Texas, USA

1. Introduction

The pivotal role of kinases in signal transduction and cellular regulation has lent them considerable appeal as therapeutic targets across a broad spectrum of cancers. The epidermal growth factor receptor (EGFR) was the first receptor tyrosine kinase to be discovered and remains the most investigated. Most of the mechanistic principles of receptor tyrosine kinases were first established with the EGFR family as a model. EGFR is a single pass transmembrane receptor with two extracellular, cysteine-rich regions involved in ligand binding, and intervening region important for receptor dimerization, an intracellular tyrosine kinase domain, and a number of intracellular sites for autophosphorylation, phosphorylation by other kinases, and docking of signaling components. Three additional EGFR family members have been identified, human epidermal growth factor receptor (erbB or HER) 2, 3 and 4. In many cell types, including those of epithelial and mesenchymal lineages, receptors of the HER family transduce signals from the cell surface to the intracellular domain, regulating normal cell growth, lineage determination, repair and functional differentiation. A range of growth factors serves as ligands for these receptors, although none have been identified for the HER2 receptor. Ligands for HER1/EGFR include epidermal growth factor (EGF) and transforming growth factor-α (TGF-α), and heregulins serve as ligands for both HER3 and HER4. Binding of a ligand to a HER family member leads to receptor homodimerization, or heterodimerization with another HER receptor, bringing about receptor phosphorylation. Ligand binding to HER1, HER3 or HER4 induces rapid receptor dimerization, with a marked preference for HER2 as a partner(Graus-Porta et al., 1997). Moreover, HER2-containing heterodimers generate intracellular signals that are significantly more potent than signals emanating from other HER combinations. Of the receptor dimers, HER3 homodimers cannot initiate signal transduction.The differing signaling characteristics of the HER family members are thought to be due to their different ligand-binding affinities and the type of phosphorylated homo- or heterodimer formed, and the resulting intracellular signaling events. Two of the key pathways involved in this cascade are the ras-raf-mitogen-activated protein kinase (MAPK) pathway, which affects DNA synthesis and cell proliferation, and the phosphatidylinositol 3-kinase (PI3-K)/Akt pathway, which plays a role in cell metabolism and survival (Yarden and Sliwkowski, 2001; Bange et al., 2001).

Functional dysregulation of the EGFR family is frequently observed in human cancers, suggesting that its signal modulates processes that play a pivotal role in tumorigenesis and disease progression. Activating mutations, gene amplification and overexpression of HER family kinases have been implicated as integral contributors to a variety of epithelial cancers, including breast, colon, pancreas, lung, and squamous cancers and also glioblastomas(Yarden, 2001; Foley et al., 2010; Yarom and Jonker, 2011; Wheeler et al., 2010; Dobashi et al., 2011; Machiels and Schmitz, 2011; Hwang, 2008). This is highlighted by HER1/EGFR overexpression or activation by autocrine or paracrine growth factor loops in at least 50% of epithelial malignancies, HER2 amplification and overexpression in approximately 20–25% of breast cancers and variable HER3 and HER4 expression in breast and other cancers. Overexpression and/or activation of HER1/EGFR, HER2/neu and HER3 have been correlated with poor prognosis. In contrast, it is unclear whether HER4 induces cell division or differentiation, and there is controversy regarding its role in breast cancer, with conflicting reports associating overexpression with shorter or prolonged survival (Normanno et al., 2006). Therefore, attenuation of HER family signaling is a developing strategy for the management of human malignancies and is the subject of a number of ongoing clinical trials.

Signaling from the plasma membrane by receptor tyrosine kinases (RTKs) to initiate downstream events that bring about their biological effects is well established. In the past decade, it has become increasingly apparent that several RTKs also move to several intracellular compartments, with nuclear localization in many cases being necessary for full function of the receptors. Access to the inside of the cell by the RTKs has in fact been shown to contribute to genesis and progression of a variety of human tumors (Carpenter, 2003). Accruing evidence points to a scenario where nuclear localization of EGFR in different tumor types has impact on not only the tumor grade but also on the resistance of tumors to therapies (Lo et al., 2006b; Wang and Hung, 2009). Here we critically evaluate the existing literature regarding intracellular localization of EGFR and its importance to cancer, and discuss future directions for the field. Further, the opportunity for targeting nuclear translocation of EGFR to develop therapeutics for intervention during critical stages of development and progression of neoplasms should be evaluated. There are numerous excellent recent reviews of HER family biology, signaling and therapeutic targeting (Yarden, 2001; Yarden and Sliwkowski, 2001; Foley et al., 2010; Yarom and Jonker, 2011; Wheeler et al., 2010; Hwang, 2008). This chapter will provide an overview of aberrant EGFR signaling in the biology of gliomas highlighting several key developments in the rapidly evolving area of HER family biology with specific reference to new localization of EGFR and the integral role of these receptors in malignant transformation and as targets of cancer therapy specifically in glioblastomas.

2. EGFR in glioma

Although a large number of other genetic alterations also occur in GBM, EGFR represents the most frequently amplified gene (2008; Hatanpaa et al., 2010). In fact, in primary GBM, it has been noted for about two decades and confirmed recently with two large-scale cancer genomics studies that EGFR amplification and mutation is a recurrent occurrence, being detected in about 40% of the samples (2008; Parsons et al., 2008). However, this is a rare event in low-grade gliomas (Ohgaki and Kleihues, 2007), suggesting a causal role for aberrant EGFR signaling in the pathogenesis of primary GBM. The resulting dysregulation

of the signal transduction process affects gene transcription and protein translation, stimulating tumor cell proliferation, tumorigenesis, migration, adhesion and angiogenesis, and inhibiting apoptosis (Huang et al., 2009). Recent research has shown an unfavorable prognostic relationship between EGFR amplification and overall survival in patients with GBM (Aldape et al., 2004; Heimberger et al., 2005; Pelloski et al., 2007). EGFR expression is also associated with enhanced resistance to radiation in malignant GBM cells *in vitro* and is correlated with poor radiographic response to radiation therapy in some patients with GBM (Nakamura, 2007; Hatanpaa et al., 2010). EGFR dysregulation in GBM result from a number of different mechanisms. Primarily, gene amplification causes EGFR overexpression, which correlates with disease progression, poor prognosis and reduced sensitivity to chemotherapy. In addition, overproduction of EGFR ligands, such as TGF-α and EGF can cause autocrine receptor activation, enhancing transformation and leading to independent tumor cell growth. Further, activating mutations in EGFR generates EGFR mutants that demonstrate constitutive activity resulting in activation of the downstream intracellular cascade without ligand binding and insensitivity to regulation by normal cellular controls (Hwang, 2008).

While there are various mutant forms of EGFR in GBM, the most prevalent and well studied EGFR mutation is EGFRvIII (Hwang, 2008). EGFRvIII is detected in about half of all tumors with EGFR amplification (Aldape et al., 2004; Heimberger et al., 2005). Immunohistochemical and quantitative PCR studies of human GBM biopsies suggest that in most tumors the overexpression or amplification of EGFRvIII occurs concurrently with that of EGFR. Only in about 10% of glioblastomas does overexpression or amplification of EGFRvIII exceed that of the wild-type receptor (Sugawa et al., 1990; Furnari et al., 2007). In addition to GBM, EGFRvIII has been detected in several human cancers including NSCLC and breast carcinomas (Moscatello et al., 1995; Ge et al., 2002; Ji et al., 2006; Sasaki et al., 2007). Structurally, EGFRvIII exhibits a large inframe deletion of the extracellular ligand binding domain which results from the elimination of a 801 base pair (nucleotide positions 275–1075) DNA fragment containing exons 2–7 of the gene (Sugawa et al., 1990; Ekstrand et al., 1991; Wong et al., 1992; Frederick et al., 2000). This partial deletion of the ligand-binding domain renders EGFRvIII ligand insensitive and also constitutively activated. The ability of this variant to 'switch on' cell signaling without ligand stimulation, albeit at low levels, results in increased oncogenic signaling and aggressive behavior in tumors that express it (Huang et al., 1997; Nishikawa et al., 1994; Chu et al., 1997). Further, it has a low rate of receptor endocytosis, so is not readily attenuated and/or down-regulated (Chu et al., 1997; Huang et al., 1997). EGFRvIII correlates with advanced disease and resistance to therapy (Hwang, 2008). Several studies have found that it enhances tumorigenicity in human glioma xenografts in mice. The introduction of EGFRvIII into glioma cells enhances tumorigenicity, proliferation, migration, and inhibits apoptosis (Huang et al., 1997; Nagane et al., 1996; Nagane et al., 2001). In a transgenic mouse model, EGFRvIII was found to contribute to glioma progression in the presence of other predisposing genetic changes, such as mutated RAS, but failed to initiate tumors on its own (Holland et al., 1998; Bachoo et al., 2002; Ding et al., 2003). Furthermore, there is growing evidence from *in vitro* studies in human glioblastoma cells, human tumor xenografts in animal models, and in patients with GBM, to suggest that EGFRvIII promotes tumor growth and progression in part via constitutive activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (Choe et al., 2003; Aldape et al., 2004; Mizoguchi et al., 2006; Pedersen et al., 2005; Huang et al., 2007). The role

of the mitogen-activated protein kinase (MAPK)/ERK 1/2 signal transduction pathway downstream of EGFRvIII is still unclear. Constitutive activity as well as impaired attenuation has been implicated as mechanisms for the enhancement of human glioblastoma cell tumorigenicity by EGFRvIII as compared to wild-type EGFR. Further, it has been recently demonstrated that EGFRvIII overexpression results in secretion of IL-6 and other cytokines to potentiate tumorigenesis in a paracrine manner (Inda et al., 2010). These data may explain the observation that patients with EGFRvIII-expressing tumors have a shorter interval to clinical relapse and poorer survival than patients with EGFRvIII-negative tumors. For GBM patients who survive 1 year or longer after diagnosis, the expression of EGFRvIII is also an independent negative prognostic indicator of survival (Aldape et al., 2004; Heimberger et al., 2005; Pelloski et al., 2007), which makes EGFRvIII an ideal target for antitumor therapy.

Signaling pathways are complex, multidimensional events that frequently converge, diverge, and cross talk with one another. The traditional cellular functions of EGFR and EGFRvIII has been reviewed elsewhere; this review focuses specifically on the role played by EGFR and EGFRvIII in the nuclear compartment as well as its nuclear downstream effectors in a number of human malignancies, and the possible novel therapeutic opportunities provided for development of anti-tumor agents.

3. EGFR/EGFRvIII in mitochondria

In the past decade, it has become increasingly apparent that several RTKs, including EGFR act also from intracellular organelles wherein they elicit biological effects that are uniquely distinct from their cell-surface functions. In addition to translocation to the nucleus (which is described in detail in the next sections), EGFR also localizes to the mitochondria, where it interacts with COXII and regulates cell survival in an EGFR Tyr845-dependent manner (Boerner et al., 2004). Src can activate EGFR by phosphorylating it on the Tyr845 and interestingly, the kinetics of mitochrondrial localization of both EGFR and Src (which also undergoes mitochondrial import) seem to be similar suggesting that they might translocate in the same complex (Demory et al., 2009). While the complete mechanism by which EGFR translocates to the mitochondria has not yet been defined, a recent investigation revealed that both EGFR and Src kinase activity was essential for mitochondrial translocation. Further, the same group identified that a mitochondrial localization signal in the juxtamembrane region of EGFR (residues 645 to 666) as well as the endocytic pathway are important for the movement of EGFR to the mitochondrion (Demory et al., 2009). Since the fusion of endosomes with mitochondria has not been demonstrated, the actual process by which endosomal EGFR moves to the mitochondria is still open to investigation. However, it was also shown that EGFR interacted with Tom40 (Demory et al., 2009), the pore component of the TOM complex and an outer mitochondrial membrane transport protein that is necessary for transport of newly synthesized mitochondrial proteins encoded by nuclear DNA. This suggests that EGFR could utilize the TOM complex to enter the mitochondria. Further, a report also demonstrated that EGFR translocates to the mitochondrion during autophagy where it again promoted cell survival (Yue et al., 2008), implying that mitochondrial localization of EGFR could potentially be an important survival strategy for cells. The same study showed that EGFR mitochondrial translocation can be increased by the mTOR inhibitor, rapamycin, and decreased by the topoisomerase inhibitor, etoposide, and by 3'-methyladenine, an inhibitor of autophagy. The reported link between

autophagy and mitochondrial EGFR is particularly interesting due to the recent observation that apoptotic stress induces EGFR mitochondrial transport. Given the pivotal role that mitochondria plays in intrinsic apoptosis, a study by Cao et al (Cao et al., 2011) show that not only EGFR but EGFRvIII can also translocate to the mitochondria when induced by apoptosis-inducing agents and EGFR kinase inhibitors(Iressa) and that tumor cells with accumulated mitochondrial EGFRvIII are resistant to apoptosis induced by these agents. These results implicate mitochondrial EGFR/EGFRvIII in the modulation of mitochondria-mediated apoptosis. Taken together these studies suggest that tumor cells re-program their intracellular trafficking of EGFR/EGFRvIII by increasing its mitochondrial accumulation, as a mechanism for escape from therapy- and stress-induced apoptosis and growth suppression.

4. EGFR in the nuclear compartment

In addition to ligand recognition, binding, and initiation of intracellular signaling cascades, receptor tyrosine kinases such as the EGFR family have newly emerging roles in the regulation of nuclear functions through their direct translocation to the cell nucleus. Broadly speaking two kinds of nuclear translocation have been identified: EGFR, HER2 and HER3 have been found as full-length proteins in the nuclear compartment and this will be our focus here; in contrast HER4 nuclear translocation and transactivation functions occur through a separate mechanism involving cleavage by matrix metalloprotease of the extracellular domain upon receptor activation, and cleavage of the intracellular domain by gamma secretase, releasing a protein fragment that translocates to the nucleus (Carpenter, 2003). This carboxyl-terminal HER4 fragment then associates with the WW-domain-containing transcriptional regulatory protein YAP (Yes-associated protein) and acts as a co-transcriptional activator (Ni et al., 2001). The physiological and clinical importance of nuclear EGFR has been underscored by the observation that presence of EGFR in the nuclear compartment in tumor biopsies correlates strongly with poor prognosis and resistance to standard treatment modalities (Wang and Hung, 2009).

4.1 Nuclear localization and function

Almost two decades of research have documented the presence of EGFR and its ligand EGF in the nucleus. Early studies focused on EGF localization and demonstrated that nuclear presence of intact EGF was dependent on the expression of EGFR (Rakowicz-Szulczynska et al., 1986; Rakowicz-Szulczynska et al., 1989). The use of labeled EGF enabled definitive documentation that nuclear EGF was derived from extracellularly added ligand (Rakowicz-Szulczynska et al., 1989). The same studies demonstrated that the nuclear membranes and isolated chromatin contained EGFR which was indistinguishable from plasma membrane localized EGFR both biochemically and immunologically (Rakowicz-Szulczynska et al., 1989; Oksvold et al., 2002; Cao et al., 1995; Marti et al., 1991; Marti and Wells, 2000). Further, EGF could stimulate phosphorylation of nuclear EGFR and other nuclear proteins in isolated nuclei and nuclear membranes, and could be specifically inhibited by monoclonal antibodies directed against the extracellular domain of cell surface EGFR (Holt et al., 1994).

In normal tissues, nuclear expression of EGFR has been shown to be associated with the acquisition of proliferative capabilities of the cell. In hepatocytes which start proliferating after hepatectomy, nuclear EGFR was detected. In contrast, quiescent hepatocytes did not show nuclear EGFR (Marti et al., 1991). EGFR was also identified in the nucleus of mouse

lung epithelial cells where it was shown to respond to neuregulin stimulation(Zscheppang et al., 2006). Additionally, functional nuclear EGFR was demonstrated in human placenta where its role as a transcription factor was put forth (Cao et al., 1995). Further, nuclear localization of EGFR was demonstrated in primary human umbilical venous endothelial cells (HUVEC) and arterial endothelial cells (HUAEC) derived from early fetal gestation (Bueter et al., 2006).

Three basic functions of nuclear EGFR have been defined thus far (Figure 1). They are

1. gene regulation/transactivation,
2. tyrosine phosphorylation of target proteins and
3. protein-protein interactions leading to DNA repair

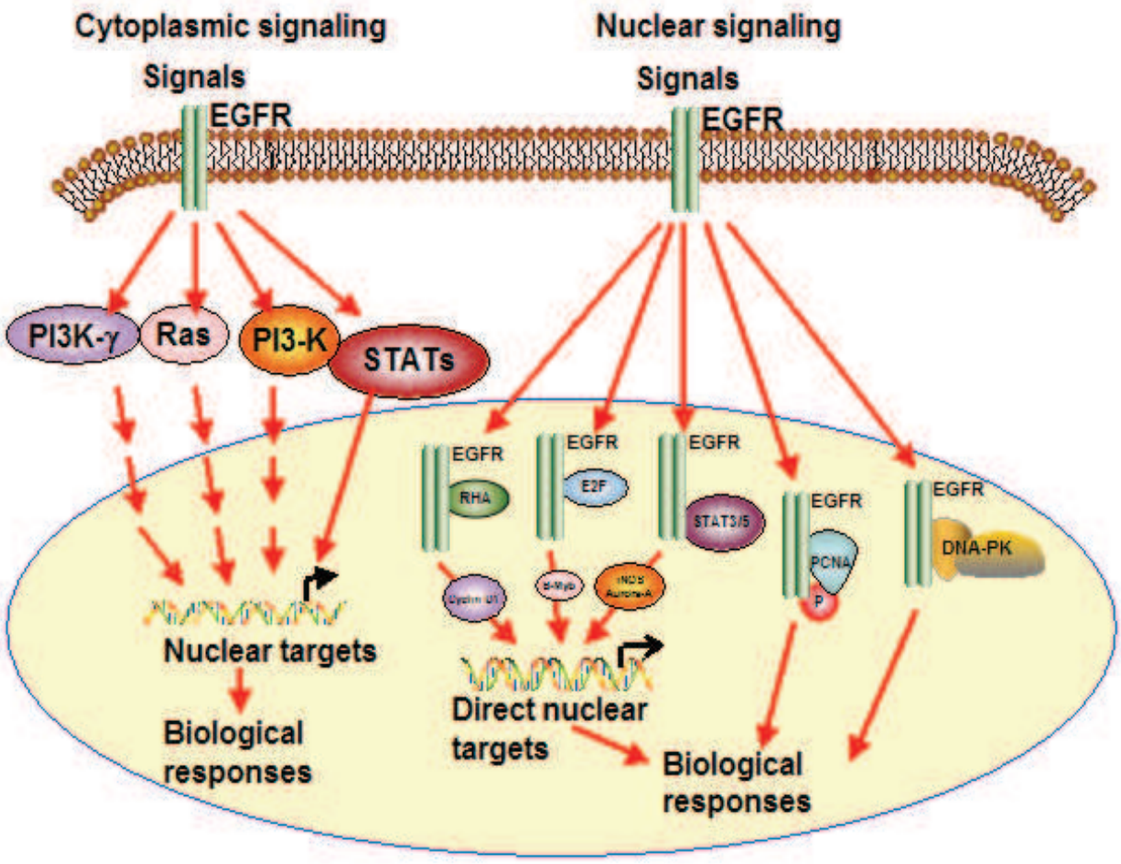

Fig. 1. Schematic representation of nuclear functions of EGFR. The cartoon shows both traditional cytoplasmic signaling eliciting nuclear effects as well as the more recently identified nuclear translocation and direct nuclear effects. Adapted from (Lo et al., 2006c)

4.1.1 Regulation of transcription

Although nuclear localization and potential transcriptional activity of EGFR had been noted in previous publications, Lin et al. demonstrated for the first time the presence of a strong transactivation domain in the C-terminal Pro-rich domain of EGFR, which mediated nuclear transactivation functions in a sequence-specific fashion (Lin et al., 2001). Although an EGFR

mutant which lacked the transmembrane domain could also translocate to the nucleus, this movement required both wild type EGFR and active ligand signaling (Marti and Wells, 2000). Upon ligand stimulation, EGFR translocated to the nucleus and associated with an A/T-rich sequence (ATRS) in the cyclin D1 promoter, resulting in transcriptional activation of cyclin D1. This study thus defined a direct link between nuclear EGFR function and cell proliferation specifically in tumors (Lin et al., 2001). Interestingly, it had been shown about a decade earlier that for induction of a mitogenic response, amphiregulin, one of the ligands of EGFR, had to undergo nuclear translocation where it bound to AT-rich DNA sequences (Kimura, 1993). Since EGFR lacks a putative DNA-binding domain, the wide-held idea in the field is that EGFR first associates with DNA-binding transcription factors and then enhances target gene transcription via their intrinsic transactivational activity. Another mechanism underlying nuclear EGFR-mediated gene regulation could be attributed to its interaction with mucin 1 (MUC1) wherein there is increased accumulation of chromatin-bound EGFR on cyclin D1 promoter and significant co-localization with phosphorylated RNA polymerase II, a marker of active transcription (Bitler et al., 2010). A recent study showed that EGFR was also able to associate with ATRS sites on DNA by association with RNA helicase A (RHA), a DNA and RNA binding protein (Huo et al., 2010). This association was found to be absolutely necessary for EGFR recruitment to the ATRS site of cyclin D1 promoter and partially necessary for recruitment onto another ATRS-containing promoter of inducible nitric oxide synthase (iNOS) gene (Huo et al., 2010). The partial role of RHA in EGFR regulation of iNOS could be because nuclear EGFR has been shown to get recruited to iNOS promoter via physical protein-protein interaction with the well characterized transcription factor, signal transducer and activator of transcription 3 (STAT3) (Lo et al., 2005a). Physical interaction and functional cooperation of EGFR with STAT3 at the cytoplasmic level is very well characterized. According to this classical model, active EGFR at the plasma membrane binds to the SH2 domain of STAT3, via the two docking autophosphorylated tyrosines (Tyr1068 and Tyr1086)(Shao et al., 2003). This interaction leads to the phosphorylation of STAT3 at Tyr705 and its activation. The dimeric STAT complex translocates to the nucleus, where it binds to specific DNA promoter sequences, recruits other transcriptional regulators, and activates the transcription of multiple genes important for cell survival (Shao et al., 2003). However, data now show that EGFR can interact with STAT3 in the nucleus where they form a complex that functions as a transcriptional activator for the iNOS gene (Lo et al., 2005a) and the Cox-2 gene (Lo et al., 2010), allowing for the possibility that STAT activation is not an exclusively plasma membrane/cytosolic event. Further, iNOS gene expression was also induced by nuclear EGFR in reactive astrocytes of human glaucomatous optic nerve head, implying an important role for nuclear EGFR in other diseases besides cancers (Liu and Neufeld, 2003). Furthermore, cooperation of nuclear EGFR with the transcription factor E2F1 activates expression of B-Myb, a positive regulator of G1/S cell-cycle progression (Hanada et al., 2006). Thus, B-Myb joins cyclin D1 as another transcriptional target of nuclear EGFR that promotes cell proliferation.

Another member of the STAT family that associates with EGFR in both the cytoplasmic and nuclear compartment is STAT5. There are two STAT5 proteins, STAT5A and STAT5B, which are encoded by two distinct but closely related genes. STAT5A has a higher DNA-binding affinity than STAT5B, and it is mostly involved in prolactin-directed mammary gland maturation (Grimley et al., 1999), whereas STAT5B is believed to be involved in the response

to the growth hormone. Similar to STAT3, unphosphorylated STAT5 is present in the nucleus and cytoplasm, and it constitutively shuttles in and out of the nucleus (Zheng et al., 2002). The N terminus is required for the constitutive nuclear import of STAT5, although the precise nature of its nuclear import domain remains to be determined. Following tyrosine phosphorylation, STAT5 accumulates in the nucleus where it induces its biological effects by transcriptional control of target genes (Debierre-Grockiego, 2004). As observed with STAT3, EGFR interacts with STAT5 on the ATRS motif to transactivate the Aurora-A promoter . Increased expression of the Aurora-A gene induced centrosome amplification and microtubule disorder (Hung et al., 2008). Further, studies from our laboratory have shown that EGFR can also associate with STAT5b to regulate Bcl-XL expression (Latha et. al., manuscript in preparation). Our study also showed a significant correlation between phosphoSTAT5b and EGFR expression in human GBMs. Furthermore, semi-quantitative scoring showed that patients whose tumors had detectable phosphoSTAT5 had a worse clinical outcome when compared to patients whose tumors had no detectable phosphoSTAT5 (Latha et. al., manuscript in preparation).

4.1.2 Nuclear substrates of EGFR

That the nucleus is an important site of direct action on the part of EGFR is supported by studies showing that exogenously added EGF can stimulate autophosphorylation of the EGFR, along with stimulation of phosphorylation of other non-EGF-binding proteins in isolated nuclei and nuclear membranes (Holt et al., 1994). Further, use of monoclonal antibody directed against the extracellular domain of EGFR inhibited the phosphorylation induced by EGF in isolated nuclei (Holt et al., 1994; Cao et al., 1995). Thus, apart from functioning as a transcriptional coregulator to modulate gene expression, the regulated kinase activity of EGFR in the nucleus can control the activation state of nuclear factors and thereby gene expression as well as other functions of such nuclear factors. The only direct nuclear substrate identified thus far is proliferating cell nuclear antigen (PCNA) (Wang et al., 2006). Wang et al. showed that nuclear EGFR phoshorylates chromatin-bound PCNA on Tyr211, and this phosphorylation is required for maintaining PCNA function on chromatin. Inhibition of the phosphorylation led to degradation of the chromatin-bound, but not the unbound, form of PCNA in a proteasome-dependent manner and consequently suppressed its function in DNA synthesis and DNA damage repair (Wang et al., 2006). This important finding raised the possibility that additional nuclear proteins may be phosphorylated by nuclear EGFR and their functions, stability, and/or subcellular localization altered as a consequence of tyrosine phosphorylation. Future efforts are needed to explore this possibility.

4.1.3 Regulation of DNA repair by nuclear EGFR

In addition to ligand-induced accumulation of EGFR in the nucleus, EGFR has been demonstrated to escape receptor degradation after internalization and translocate to the nucleus in response to stress signals such as ionizing radiation, heat shock, H_2O_2, and cisplatin (Rodemann et al., 2007). Specifically, ionizing radiation causes nuclear transport of EGFR via caveolin and protein kinase C dependent mechanisms (Khan et al., 2006). Further, radiation has also been shown to drive EGFR translocation from the perinuclear region to the nucleoplasm via a process involving generation of free radicals (Dittmann et al., 2005a). Also, ultraviolet irradiation has been shown to induce EGFR nuclear translocation in human

keratinocytes (Xu et al., 2009). In addition, Bandyopadhyay et al. reported that nuclear EGFR can influence DNA repair directly via physical interaction with DNA-dependent kinase (DNA-PK). They showed that cetuximab, an EGFR neutralizing antibody could decrease nuclear DNA-PK protein and kinase activity (Bandyopadhyay et al., 1998). Cetuximab treatment was also shown to block nuclear shuttling of EGFR and increased sensitivity to radiation-induced cell death in these cells (Dittmann et al., 2005b). It was also shown that the presence of EGFR in the nucleus resulted in activation of DNA-PK as shown by its phosphorylation on Thr2609. In the same study, it was again demonstrated that nuclear EGFR binds to the catalytic subunit DNA-PKs, and the regulatory subunit Ku70 of DNA-PK (Dittmann et al., 2005b). Nuclear EGFR has been shown to associate with p53 and MDC1 protein, an essential protein for the recruitment of DNA repair foci, following irradiation and treatment with the radioprotector Bowman-Birk proteinase inhibitor (Gueven et al., 1998; Dittmann et al., 2008a). Another radioprotector O-phospho-l-tyrosine (P-Tyr) has been shown to activate PKCepsilon, which triggers nuclear EGFR accumulation and concurrent phosphorylation of DNA-PK at amino acid residue Thr2609, and leads to repair of DNA double-strand breaks (Dittmann et al., 2007). Together these observations suggest an important role of nuclear EGFR for regulation of DNA repair following treatment with genotoxic substances.

4.2 Mechanism of nuclear transport

The intact EGFR protein containing both intracellular and extracellular domains moves to the nucleus in a ligand-bound form and the mechanism of this translocation is still incompletely understood. A seminal study very clearly demonstrated that intact EGFR bound by monoclonal antibodies directed against extracellular residues can accumulate specifically in the nucleus only in EGFR-expressing cells (Holt et al., 1994). This provided the first clue that endocytic events associated with the plasma membrane receptor could lead to intracellular trafficking and the delivery of EGFR to the nucleus. Further studies have convincingly demonstrated that a novel pathway exists by which internalized EGFR in the early endosomes is transferred into the nucleus instead of lysosomes or being recycled to the cell surface. Inhibition of receptor endocytosis using a dominant-negative dynamin mutant, which abrogates the formation of clathrin-coated pits from the cell surface, blocks the nuclear transport of EGFR (Lo et al., 2006a). Further, EGFR has been shown to colocalize with EEA1, an early endosomal marker in the nuclear compartment (Giri et al., 2005; Lo et al., 2006a). Thus, it seems clear that EGFR utilizes the endocytic pathway for its translocation into the nucleus, though additional routes cannot be excluded.

Nuclear localization requires recognition by the cellular nuclear import machinery and, in particular, by members of the nuclear localization sequence (NLS)-recognizing IMP superfamily. The best-understood NLS dependent nuclear import pathway is that mediated by the IMPα/β heterodimer. After irradiation, it was demonstrated that EGFR is found in complex with Importin α and RAN-GTP. It was also shown that EGFR-dependent activation of the PI3K/AKT signaling pathway was important for this association (Dittmann et al., 2005a). A prerequisite for Importin binding is the presence of an NLS within the cargo protein. EGFR has been shown to contain a tripartite NLS in its juxtamembrane region that mediates the nuclear translocation. EGFR has also been demonstrated to interact with importins α1/β1 for which the NLS appears to be indispensable (Lo et al., 2006a; Lin et al., 2001; Hsu and Hung, 2007). Interestingly, another group studying nuclear transport of

EGFR following irradiation observed phosphorylation of EGFR at residue T654, which is located within the EGFR NLS. Furthermore, the same group identified PKCepsilon as the kinase responsible for this modification (Wanner et al., 2008). Nuclear EGFR accumulation results from a balance of import and export processes. Recent evidence also showed that the CRM1 is involved in nuclear–cytoplasmic shuttling of EGFR (Lo et al., 2006a). Existence of nuclear export sequences within EGFR sequence, however, has not yet been demonstrated.

While the above delineated mechanisms shed light on the mechanics of nuclear translocation of EGFR, it still is unclear how a transmembrane receptor is processed into a nuclear non-membrane-bound receptor. A protein translocon mediates the trafficking of protein complexes into and out of lipid bilayers. It was first suggested by Liao & Carpenter that the Sec61 translocon could mediate nuclear transport of EGFR (Liao and Carpenter, 2007). The Sec61 translocon is located in the endoplasmic reticulum and mediates the trafficking of secretory and transmembrane proteins into the ER during protein sythesis. This translocon is bidirectional and functions also, as part of the ERAD pathway, to retrotranslocate malfolded transmembrane proteins from the ER to the cytoplasm. Studies showed that radiation and EGF induced trafficking of EGFR to the ER membrane presumably via membrane fusion of the late edosomes to the golgi apparatus where it interacted with the Sec61 translocon. The data showed that knock-down of a Sec61 subunit abrogates both EGFR nuclear localization and EGF induction of cyclin D1 (Liao and Carpenter, 2007). While it has been proposed that Sec61 associated EGFR is retrotranslocated to the cytoplasm and subsequently imported into the nucleus via Importinβ and the nuclear pore complex (NPC), a recent study showed that Sec61β present in the inner nuclear membrane (INM) mediates retrotranslocation of EGFR from the INM into the nucleus (Wang et al., 2010b). Since the ER membrane system is contiguous with the outer nuclear membrane (ONM) and it is known that the ONM connects to the INM at the NPCs, it presents a model where EGFR is traficked first to ER via the endocytic machinery, then to the INM and then released into the nucleus by retrograde translocation at the NPC (Figure 2).

4.3 Nuclear EGFR in the clinical setting

Over the last 20 years, multiple reports have demonstrated beyond doubt that EGFR can translocate into the nuclear compartment where it has specific functions as outlined above. Concurrent studies have also identified that nuclear localization of EGFR is a common component of numerous cancer types. In addition to rapidly proliferating cells, nuclear EGFR has been detected in glioma as well as cancers of the breast, epidermoid, bladder, ovary, and oral cavity (Wang and Hung, 2009).

Increased nuclear EGFR expression is a prognostic feature in multiple tumor types correlating with parameters of known prognostic significance, such as tumor grade, stage and metastasis and is also associated with resistance to standard therapy such as radiation and cisplatin (Wang and Hung, 2009; Hwang, 2008). Analysis of nuclear localization of EGFR in human breast carcinoma biopsy samples demonstrated that nuclear EGFR levels inversely correlated with survival and correlation existed between nuclear localization of EGFR and markers of proliferation such as Cyclin D1 (that was shown to be a transcriptional target of EGFR in the same study) and Ki-67 (Lin et al., 2001). A follow up study also showed positive correlation between nuclear EGFR, Cyclin D1 and RHA expression in breast tumors (Huo et al., 2010). Early investigations revealed that cases with high nuclear EGFR demonstrated a trend towards poor survival in oral carcinomas (Lo et al., 2005b). This

was later corroborated by studies that showed that nuclear EGFR was associated with increased local recurrence rate and inversely correlated with disease free survival (Psyrri et al., 2005). A study in esophageal squamous cell carcinoma using antibodies specific for phosphorylated EGFR showed that pEGFR not only localized to the nucleus but also correlated with higher TNM stage, nodal metastasis, and poor patient outcome (Hoshino et al., 2007). A follow up analysis by the same group showed that there was a strong correlation between nuclear EGFR and PCNA as well as nuclear ERK2 and non-nuclear EGFR (Psyrri et al., 2008). The above study was in line with earlier findings that PCNA is a substrate for EGFR in the nucleus and PCNA Tyr211 phosphorylation was associated with pronounced increase in cell proliferation and worse survival of breast cancer patients (Wang et al., 2006). A recent study also demonstrated that an inverse correlation exists between increased nuclear localization of EGFR and overall survival in ovarian tumor samples (Xia et al., 2009).

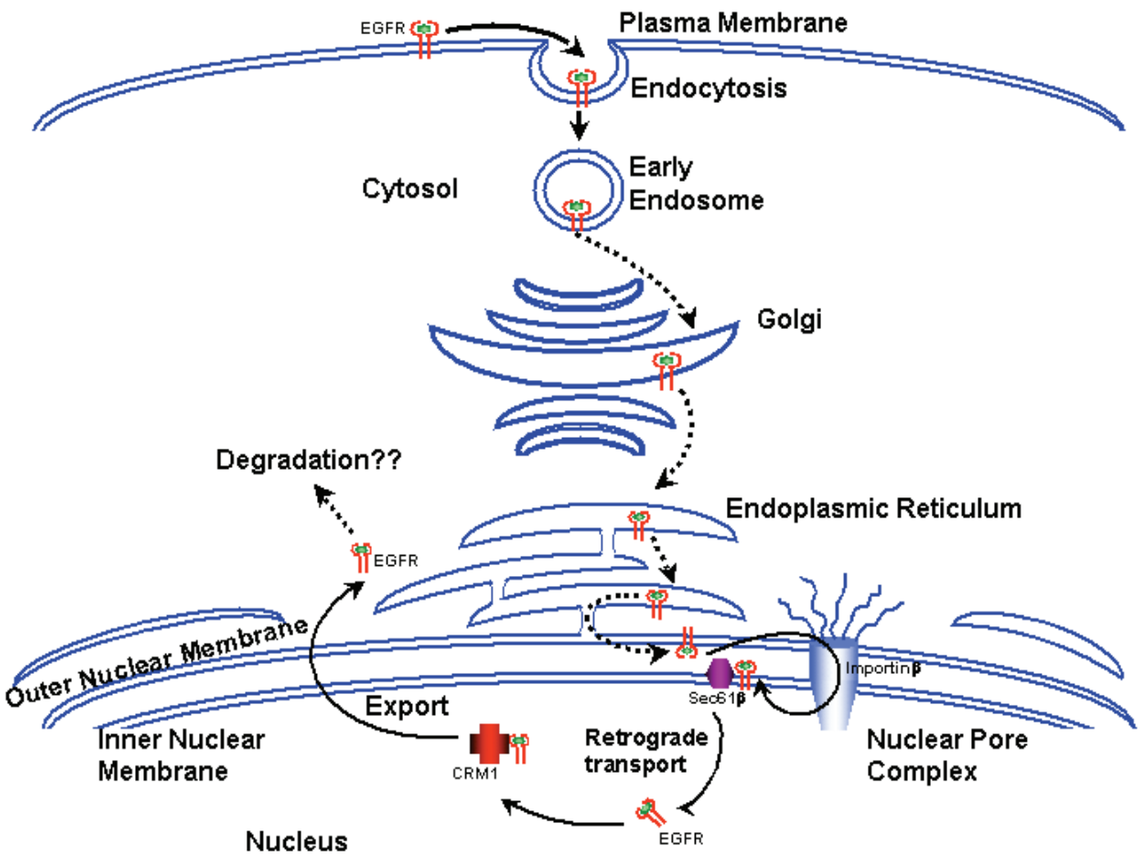

Fig. 2. A cartoon depicting the transport of EGFR from the plasma membrane to the nucleus. The current model shows the role of endocytosis for movement of EGFR to the early endosome, Golgi and thereafter, to the ER. From the ER, it is assumed that EGFR moves to the ONM and then via the NPC and Importinβ to the INM. On INM, it associates with Sec61β and through retrograde transport is released into the nucleoplasm. Nuclear export occurs via CRM1. The dotted lines represent potential pathways while solid lines depict pathways that have been experimentally proved. After (Wang et al., 2010a)

These clinical correlations of nuclear EGFR expression underscore the importance of examining the role of nuclear EGFR in other cancers especially those of epithelial origin as well as other human diseases. In this context, interestingly in addition to cancers, localization of EGFR in the nuclear compartment has also been implicated in glaucoma and neuropathy of the optic nerve (Liu and Neufeld, 2003). While the above studies indicate that nuclear EGFR is a prognostic indicator for poor clinical outcome and nuclear expression of EGFR is associated with cancer development, all the studies have been correlative studies. Mechanistic investigations delineating oncogenic capacity of nuclear EGFR are still limited and need to be further addressed. Further, a systematic approach has, yet to be conducted to identify nuclear EGFR target genes. Knowledge of the target genes will advance our understanding of the nature and effect of nuclear EGFR on cell physiology, provide novel insights into the role of nuclear EGFR in human cancers, and help clarify some of the molecular mechanisms underlying the observed association between nuclear EGFR and poor clinical outcome.

5. Role of EGFRvIII in the nucleus

A substantial body of evidence suggests that the EGFRvIII contributes a strong oncogenic signal in GBM while simultaneously protecting them from conventional therapy (Hwang, 2008; Gan et al., 2009). Two recent reports briefly described the nuclear localization of EGFRvIII in glial cells as well as glioblastoma (de, I et al., 2008; Lo et al., 2010). In glioblastoma, nuclear EGFRvIII was found to form a complex with STAT3 as was observed for EGFR. The EGFRvIII-STAT3 oncogenic complex was found to be essential for EGFRvIII induced transformation of astrocytes (de, I et al., 2008). Further, the second report of EGFRvIII nuclear localization showed that the EGFRvIII-STAT3 transcriptional complex could get recruited onto the promoter of Cox2 gene in glioma cells and increase transcription of Cox2 (Lo et al., 2010). Likewise, our finding that EGFRvIII binds STAT5b and positively regulates the transcription of Aurora A and Bcl-XL is the first demonstration of a role for EGFR/STAT5 pathway in the nucleus. Importantly, we show that nuclear EGFRvIII cooperates with STAT5b to regulate the Bcl-XL promoter and its expression in gliomas (Latha et. al., manuscript in preparation). This suggests that nuclear EGFR and EGFRvIII may be acting in concert with several members of the STAT family to generate a specific facet of its oncogenic signal in the nucleus and they also provide a potential role for combination therapy targeting EGFR/EGFRvIII and STAT5.The above studies suggest that the EGFRvIII-STAT family axis is important in gliomas. Although the above studies have shown nuclear translocation of EGFRvIII, the mechanism of translocation as well as the oncogenic role of EGFR in the nucleus remains unkown. Our recent investigation has shown that in gliomas, nuclear EGFRvIII as well as EGFR is membrane-derived and cycling of EGFRvIII and EGFR between the cytoplasm and nucleus is dependent on Importinβ and CRM1. Moreover, by manipulating EGFRvIII such that it is restricted to either the nucleus or the cytoplasm followed by functional studies, we show that nuclear EGFRvIII drives tumor formation. There is dramatic attenuation in the tumorigenic capacity of EGFRvIII upon blockade of its nuclear translocation in glioma cells (Gururaj et. al., manuscript in preparation). This suggests that while only a fraction of EGFRvIII or EGFR is in the nucleus, signaling here may be particularly important as it offers direct access to transcriptional regulators such as members of the STAT family. Taken together, these data provide an

important insight into a new signaling node that may make a unique and key contribution to the oncogenic signal from overactive and mutant EGFR.

Nuclear expression EGFRvIII has also been described in prostate cancer (Edwards et al., 2006) and invasive breast cancer. In hormone-refractory prostate cancer, nuclear expression of EGFRvIII was associated with decreased time to death from relapse and decreased overall survival (Edwards et al., 2006). EGFRvIII is associated with radioresistance as well as chemoresistance (Huang et al., 2009; Schmidt and Lichtner, 2002; Chakravarti et al., 2004), which may be related to nuclear localization, in light of the finding that EGFR's association with nuclear targets is key to its ability to protect cells from DNA damaging agents, discussed above. *In vitro* studies using established glioma cell lines have shown that EGFRvIII confers resistance to cisplatin (Nagane et al., 1998; Nagane et al., 2000; Lammering et al., 2001; Lammering et al., 2003; Stea et al., 2003) and IR . Further, xenograft studies have shown that EGFR-specific inhibitors (small-molecule as well as α-EGFR antibodies) significantly enhance the efficacy of radiotherapy (Chakravarti et al., 2004; Harari and Huang, 2000). Also, EGFRvIII expression has been shown to enhance radioresistance by promoting the rapid repair of radiation-induced DNA double-strand breaks (Mukherjee et al., 2009). In line with this, a recent study showed that as with EGFR, EGFRvIII associates with DNA-PKcs after radiation and cisplatin treatment. They also showed that EGFRvIII not only conferred resistance to the two treatment modules but also significantly increased the activity of DNA-PKcs in cells (Liccardi et al., 2011). Recently, two independent studies showed that nuclear EGFR contributes to resistance to cisplatin treatment and cetuximab treatment (Hsu et al., 2009; Li et al., 2009). One of the studies also demonstrated that nuclear EGFR is required for DNA repair (Hsu et al., 2009). However, the role of nuclear EGFRvIII in either chemo- or radiation resistance in GBM is yet to be explored.

6. Nuclear EGFR/EGFRvIII and therapeutics

Since EGFR function is aberrantly regulated in a majority of the different epithelial cancers, attenuation of EGFR signaling is one of the key strategies for the management of human malignancies. Various therapeutic strategies have been developed to block EGFR signaling, with the most frequent strategies involving humanized monoclonal antibodies directed against EGFR, and small molecule tyrosine kinase inhibitors that target the enzymatic activity of the tyrosine kinase domain.

Anti-receptor antibodies including cetuximab bind to the receptor extracellular domain and induce internalization and degradation, thus effectively blocking receptor activation of subsequent cellular signaling cascades. Cetuximab is currently used in combination with chemotherapy for treatment of metastatic colorectal cancers with EGFR expression and wt-KRAS genotype as well as recurrent or metastatic head and neck squamous cell carcinoma (HNSCC). It is also indicated in treatment along with radiation for locally advanced HNSCC. As monotherapy, it is used in treatment of patients who have failed platinum based therapy of metastatic colorectal cancers. With respect to nuclear translocation of EGFR and cetuximab, there are conflicting data. In the context of radiation induced nuclear translocation, it was shown that cetuximab treatments results in accumulation of cytoplasmic EGFR and blockade of EGFR transport into the nucleus following radiation (Dittmann et al., 2005b). In contrast, a more recent study showed that cetuximab, like EGF, can induce nuclear localization of EGFR (Liao and Carpenter, 2009). However, it is to be noted that in this study, it was also shown that while EGF induced translocation required

kinase activity, cetuximab induced translocation was kinase independent (Liao and Carpenter, 2009). This could be due to a fundamental difference either in the trafficking or conformation of EGF- and cetuximab- bound EGFR and warrants further investigation. Of interest is another study demonstrating that cells which acquire resistance to cetuximab show increased localization of EGFR in the nuclear compartment (Li et al., 2009). Further, expression of EGFR tagged with NLS that show increased nuclear localization render cells resistant to cetuximab treatment both *in vitro* and in xenograft studies (Li et al., 2009). Collectively, these data suggest that the mechanisms of the effect of cetuximab on EGFR nuclear transport are likely to be complex and dependent on cellular context. Further research is necessary to clarify the role and effect of cetuximab in nuclear translocation of EGFR. Interestingly, a recent study also showed that a monoclonal antibody which recognizes EGFRvIII and activated EGFR, Mab 806, and which has been demonstrated to inhibit the growth of tumors expressing EGFRvIII or overexpressing EGFR, can sensitize radioresistant glioma cells expressing EGFRvIII (Johns et al., 2010). These findings provide a strong rationale for the study of nuclear EGFRvIII in resistance to therapy which has not been addressed thus far.

Small molecule inhibitors such as erlotinib act against the cytoplasmic tyrosine kinase domain and inhibit the enzymatic activity of EGFR and so cytoplasmic signaling. Erlotinib is used as monotherapy for locally advanced or metastatic non-small cell lung cancer patients who have failed chemotherapy regimen and also for pancreatic cancer patients who have advanced local disease, metastasis or non-resectable tumors. However, there is insufficient evidence for using combinations of erlotinib with either chemotherapy or radiotherapy in patients and needs to be further investigated. Lapatinib, a dual EGFR and HER2 tyrosine kinase inhibitor, can sensitize cells to fluoropyrimidines possibly by inhibiting nuclear translocation of both EGFR and HER2 (Kim et al., 2009). Also, a recent study showed that nuclear EGFR is required for tumor resistance to DNA damage induced by the DNA alkylator, cisplatin (Hsu et al., 2009). Interestingly, a COX2 inhibitor, celecoxib has been shown to radiosensitize tumor cells by inhibiting radiation-induced nuclear EGFR translocation and DNA repair. Since this action of celecoxib did not correlate with COX2 expression, it is suggested that it could be a COX2 independent mechanism (Dittmann et al., 2008b). Another investigation demonstrated that the Src family kinase inhibitor, dasatinib could resensitize cells that were cetuximab resistant by inhibiting nuclear transport of EGFR (Li et al., 2009). While the above studies encourage the use of combination therapy to address treatment resistance by keeping EGFR out of the nucleus, we must proceed with caution since we are yet to understand the nuclear functions of EGFR in a comprehensive manner. Furthermore, such knowledge holds out the hope of leading to new targets that could be exploited in new combinations in the future.

7. Conclusion

Recent research has further strengthened the case for an important role of the EGFR family in a variety of cancers. It has also broadened our understanding of where these proteins may be active, by showing that in addition to propagating cytoplasmic signaling events, EGFR can also control other functions directly by translocating into the nuclear or mitochondrial compartment. Direct action within the nucleus could be a means by which the receptor achieves selectivity, specificity, and efficiency of gene activation not possible through shared transcription factors and second messenger/phosphorylation cascade systems. It may also

represent an ancient signaling connection between the limiting membrane and the genetic material and other vital cellular complexes that predate the evolution of intracellular, membrane bound organelles. Understanding the nuclear functions of EGFR as well as the mechanisms of its nuclear trafficking provides the hope of novel avenues for therapeutic interventions in various tumor types. Further, nuclear EGFR itself could be a new clinically relevant target with the potential to find application specifically in chemo- and radio-resistant tumors. However, to evaluate the fitness of nuclear EGFR as a target, a selective inhibitor of EGFR transport needs to be identified. Thus, the current challenge in the field is to gain substantial knowledge about the functions as well as translocation of EGFR and define its role in cancer prognosis, targeting, and therapeutic sensitization.

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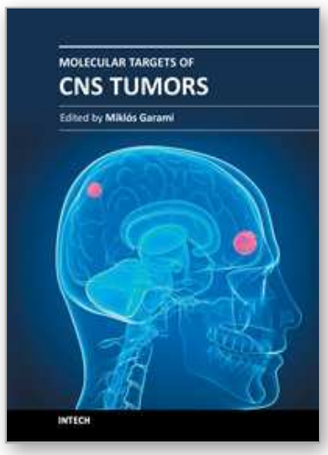

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Molecular Targets of CNS Tumors is a selected review of Central Nervous System (CNS) tumors with particular emphasis on signaling pathway of the most common CNS tumor types. To develop drugs which specifically attack the cancer cells requires an understanding of the distinct characteristics of those cells. Additional detailed information is provided on selected signal pathways in CNS tumors.

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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
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Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
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