Nuclear EGFR as Novel Therapeutic Target

Insights into Nuclear Translocation and Function

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Emerging evidence suggests the existence of a new mode of epidermal growth factor receptor (EGFR) signaling in which activated EGFR undergoes nuclear translocation following treatment with ionizing radiation. The authors provide evidence that the nuclear EGFR transport is a stress-specific cellular reaction, which is linked to src-dependent EGFR internalization into caveolae. These flask-shaped pits can fuse with endoplasmic reticulum and the EGFR is sorted into a perinuclear localization. This compartment may serve as a reservoir for nuclear EGFR transport which is regulated by PKCε (protein kinase Cepsilon). Nuclear EGFR is able to induce transcription of genes essential for cell proliferation and cell-cycle regulation. Moreover, nuclear EGFR has physical contact with compounds of the DNA repair machinery and is involved in removal of DNA damage. Anti-EGFR strategies target radiation-associated EGFR nuclear translocation in different manners. EGFR-inhibitory antibodies, i.e., cetuximab (Erbitux®), can block nuclear translocation by EGFR immobilization within the cytosol in responder cell lines, whereas tyrosine kinase inhibitors rather target nuclear kinase activity of EGFR linked with cytosolic or nuclear functions. However, both strategies can inhibit DNA repair following irradiation.

Key Words: EGFR · Nuclear translocation · DNA repair · Cetuximab · Tyrosine kinase inhibitor

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Der nukleäre EGFR als neues therapeutisches Ziel. Einsichten in die nukleäre Translokation und Funktion

Der EGFR wird als membranständiger Wachstumsfaktor-Rezeptor beschrieben. Neue Erkenntnisse zeigten jedoch, dass der EGFR z. B. nach Bestrahlung auch im Zellkern gefunden werden kann. Der Kerntransport des EGFR wird vor allem nach Stressexposition der Zelle beobachtet und ist mit einer Src-Kinase-abhängigen Internalisierung des EGFR in das endosomale Kompartment der Caveolae assoziiert. Nach Verschmelzung der Caveolae mit der Membran des endoplasmatischen Retikulums reichert sich der EGFR perinukleär an. Der perinukleäre EGFR-Pool dient wahrscheinlich als Reservoir für den Kerntransport, der nach Strahlenexposition durch die Aktivität der PKCε (Proteinkinase Cepsilon) reguliert wird. Der nukleäre EGFR agiert zum einen als Transkriptionsfaktor und induziert die Transkription von zellzyklus- und proliferationsrelevanten Proteinen, zum anderen hat er physikalischen Kontakt zu für die DNA-Reparatur essentiellen Proteinen. In der Radioonkologie finden prinzipiell zwei Anti-EGFR-Therapien Verwendung. Antikörperstrategien, z. B. die Behandlung mit Cetuximab (Erbitux®), können in sensitiven Tumorzellen zu einer Immobilisierung des internalisierten EGFR in den Caveolae führen. Die Translokation in den Zellkern ist blockiert. Im Gegensatz dazu verhindern Kinaseinhibitoren die strahleninduzierte Kerntranslokation des EGFR nicht, hemmen aber die EGFR-Kinaseaktivität und blockieren so das nukleäre und zytoplasmatische „Signaling" des Rezeptors. Auf diese Weise können beide Strategien die Reparatur von DNA-Schäden behindern und den Erfolg einer radioonkologischen Behandlung verbessern.

Schlüsselwörter: EGFR · Nukleäre Translokation · DNA-Reparatur · Cetuximab · Tyrosinkinaseinhibitor

The Outstanding Role of EGFR

A high proportion of human tumor cells is characterized by overexpression of epidermal growth factor receptor (EGFR), a protein that promotes resistance to chemo- and radiotherapy [13, 19, 31, 50, 54, 58]. EGFR protein can be activated through phosphorylation at specific amino acid residues in response to ligand binding (EGF, tumor necrosis factor-[TGF-]α and amphiregulin) [18, 65] as well as after exposure to a variety of unspecific stimuli like ionizing radiation [52], UV radiation [29], hypoxia [45], hyperthermia [17], oxidative stress [28], and transactivation by G-protein-coupled receptors [6]. Both ligand-dependent as well as ligand-independent

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phosphorylations of EGFR result in receptor internalization [60] and intracellular signaling [13, 50, 51, 57, 59]. To date, internalization is assumed to be essential for receptor silencing and inactivation. Indeed, EGF treatment results in internalization of EGFR into coated pits followed by receptor degradation [56]. Cell exposure to oxidative stress can lead to internalization of EGFR into caveolae, however, this process is associated with perinuclear accumulation of EGFR and persistent kinase activity, as reported by Khan et al. [28]. The broad inducibility of EGFR activation and internalization by cellular stress suggest an essential role of EGFR during regulation of cellular survival. The special role of nuclear EGFR has been underlined by the clinical observation, that detection of nuclear EGFR in tumor biopsies is strongly correlated with treatment resistance and poor prognosis [2, 23, 24, 31, 48, 49, 64].

Radiation-Induced Internalization of EGFR

A characteristic compound of caveolae is the protein caveolin. Caveolin gene family consists of three members: CAV1, CAV2, and CAV3, coding for the proteins caveolin-1, caveolin-2, and caveolin-3, respectively. Caveolins associate with cholesterol and sphingolipids in specific areas of the cell membrane to form flask-shaped pits called caveolae. Caveolae are involved in receptor-independent endocytosis and intracellular signaling [43]. In addition, caveolin-1 is a transmembrane protein and an essential component during interactions of integrin receptors with cytoskeleton-associated molecules [12]. Caveolae contain a high variety of proteins essential for signaling. Caveolae and associated proteins form the so-called caveosome, which can fuse with the early endosomes [3]. Moreover, caveolin-1 is found at different intracellular locations. Variations in subcellular localization are associated with a plethora of ascribed functions for this protein. These observations suggest a general function of caveolae as an intracellular signaling platform.

In agreement with that, compartmentation into caveolae prevents EGFR degradation and simultaneously enables intracellular EGFR kinase-linked signaling [28]. These findings suggest a new function of EGFR – depending on its intracellular localization –, which supplements its functions described so far and defines a new therapeutic target.

Ionizing radiation results in fast src kinase stabilization, activation and subsequent src-mediated caveolin-1 Y14 and EGFR Y845 phosphorylations. Both phosphorylations are stress-specific and cannot be observed after treatment with EGF [14], which suggests caveolae sorting of EGFR as a stress-associated event. Treatment with the EGFR-inhibitory antibody cetuximab results in some tumor cells in a strong accumulation of caveolin/EGFR complexes within cytoplasm. Radiation-induced caveolin-1 and EGFR phosphorylations are associated with nuclear EGFR transport [14, 32]. As shown by the src-specific inhibitor PP2, blockage of src activity inhibits caveolin-1 phosphorylation and decreases nuclear transport of EGFR [14].

Translocation of EGFR from Caveolae into Endoplasmic Reticulum

Nuclear localization of the EGFR requires endocytosis and association of the receptor with the karyopherin carrier nuclear import system [32]. However, this association does not explain how a transmembrane receptor is processed into a nuclear non-membrane-bound receptor. As cells do have protein complexes that translocate proteins into and out of lipid bilayers [63], Liao & Carpenter [32] explored the possibility, that the Sec61 translocon could mediate nuclear transport of the EGFR. EGFR located within the membrane of late endosomes is transferred to the membranes of Golgi apparatus by membrane fusion and at least locates in the endoplasmic reticulum (ER) membrane. For nuclear transport EGFR has to be set free from ER membrane to become a cytosolic protein and to admit access of the karyopherin system to the intrinsic nuclear localization site (NLS) of the EGFR. Indeed, the EGFR is found in complex with Sec61 following irradiation. The Sec61 translocon is located exclusively in the ER and ER/Golgi transitional region [20] and functions to insert secretory and transmembrane proteins into the ER during protein synthesis [26]. This translocon is bidirectional and also retrotranslocates proteins from ER membrane to the cytosol.

EGFR Transport into Nucleus

Passage through the nuclear pore complex needs binding to nuclear transport receptors. Many proteins are imported via karyopherin β (often using karyopherin α as an adapter). Indeed it was shown, that after irradiation the EGFR is found in complex with karyopherin α and RAN-GTP [13]. Prerequisite for karyopherin binding is the presence of an NLS within the cargo protein. Classic NLSs contain one or two clusters of basic residues. Monopartite NLSs have a single cluster of four to five basic residues, whereas bipartite NLSs are characterized by a second basic cluster located about ten to twelve residues downstream of the first cluster [16]. Molecular recognition of NLSs is essential for the formation of the import complex. Lin et al. [34] reported identification of a putative NLS within the EGFR sequence and proved the function. Interestingly, we observed phosphorylation of EGFR at residue T654, which is located within this putative EGFR NLS, after radiation-induced nuclear EGFR transport. Furthermore, we identified PKCε (protein kinase Cepsilon) as the kinase responsible for this modification [62]. Nuclear EGFR accumulation results from a balance of import and export processes [13]. Recent evidence suggests, that nuclear export of EGFR may involve exportin CRM1 [21]. Existence of nuclear export sequences within EGFR sequence, however, has not been demonstrated.

Function of Nuclear EGFR

Nuclear EGFR detection was first reported in hepatocytes that underwent regeneration and in primary adrenocortical carcinomas [38]. High levels of EGFR were detected in the

nuclei of many tumors, including those of adrenocorticord, breast, bladder, skin, thyroid, and oral cavity [35, 36, 38, 49]. Nuclear EGFR appears to be the full-length phosphorylated receptor [11, 13, 33, 34]. Nuclear EGFR positively correlates with Ki-67 expression, an indicator of proliferation [36]. Consequently, a function of nuclear EGFR as transcriptional activator was suggested. Indeed, transactivation domains within EGFR and its family members HER-2 and HER-4 were identified and found to be functional [25, 34]. Nuclear EGFR and HER-2 were shown to associate with specific DNA sequences designated AT-rich sequence and HER-2-associated sequence, respectively [25, 34]. Promoters that are targeted by nuclear EGFR are those of cyclin D1, iNOS, and B-Myb [1, 21, 34]. Given the notion that ErbB receptors lack a putative DNA-binding domain, it is suspected that these receptors first associate with DNA-binding transcription factors and then enhance target gene transcription via their intrinsic transactivational activity. In this regard, nuclear EGFR interacts with STAT3 and co-regulates iNOS expression [1]. In addition, STAT3 activation may be associated with Bcl-XL expression which can link nuclear EGFR with regulation of cell death also [27]. Furthermore, cooperation of nuclear EGFR with the transcription factor E2F1 activates expression of B-Myb, a positive regulator of G1/S cell-cycle progression [21].

The observation that nuclear EGFR is phosphorylated at autophosphorylation sites indicates that kinase activity of EGFR is present within nucleus and suggests that this kinase activity may be relevant for the function of nuclear EGFR. Indeed, Wang et al. [61] could demonstrate, that proliferating cell nuclear antigen *(*PCNA) is subject to tyrosine phosphorylation at a specific site in an EGFR-dependent manner and that this phosphorylation enhances PCNA stability on chromatin. Thus, these data link tyrosine kinase activity of nuclear EGFR with cell proliferation and DNA repair by regulating PCNA function.

In addition, Bandyopadhyay et al. [5] described that nuclear EGFR can interact with DNA repair and cell survival directly. They observed physical interaction of EGFR with DNA-dependent kinase (DNA-PK). Furthermore, they demonstrated that blocking EGFR signaling by cetuximab, an anti-EGFR monoclonal antibody, resulted in reduction of nuclear DNA-PK protein and kinase activity, implicating a role of EGFR in regulation of DNA repair. Indeed, it could be shown that nuclear EGFR is associated with phosphorylation of DNA-PK at residue T2609, which indicates DNA-PK activity during nonhomologous end-joining DNA repair [13]. Blockage of nuclear EGFR transport by cetuximab decreased DNA-PK activity and consequently increased residual DNA damage and reduced survival after radiation treatment in A549 cells [15]. These observations suggest a crucial role of nuclear EGFR for regulation of DNA repair following treatment with genotoxic substances.

Nuclear EGFR Transport: a Therapeutic Target?

As already mentioned above, increased nuclear localization of the EGFR is associated with treatment resistance and poor prognosis of tumors [23, 36, 49]. Treatment of cells either with inhibitory antibodies or tyrosine kinase inhibitors [53] are accepted strategies to counteract EGFR function [22]. As monotherapy, tyrosine kinase inhibitors are shown to be efficient in palliative second-line treatment of non-small cell lung cancer [4]. Cetuximab showed positive effects as single treatment or in combination with chemotherapy in metastatic colorectal cancer [44]. For combination treatment regimens with radiotherapy, preclinical and first clinical data report improved survival [8] and increased tumor control [7, 30, 39, 41, 42]. For use of tyrosine kinase inhibitors in combination with radiation or additional genotoxic treatments, no solid clinical trials exist so far and further clinical evaluation of this approach is necessary [9, 37, 46]. Finally, both anti-EGFR strategies seem to be effective in principle, nevertheless the molecular mode of action is different. Cetuximab binds to the extracellular part of EGFR nearby the natural ligand binding site. This binding results in a phosphorylation of the receptor associated with an internalization [47]. Interestingly, in vitro data clearly show, that in some cells cetuximab binding results in accumulation of EGFR within cytoplasm, which is associated with blockage of nuclear EGFR transport following irradiation [15]. By contrast, in other tumor cells it was demonstrated, that cetuximab treatment induced nuclear EGFR accumulation within the nucleus [33]. These contradicting data have to be resolved in additional preclinical experiments and may help to interpret heterogeneous responses of tumors upon cetuximab treatment.

In any case, the EGFR is removed from cell surface and further ligand-induced signaling is hampered [47]. By contrast, tyrosine kinase inhibitors enter the cell and block the cytosolic kinase activity of EGFR intracellularly. This means, in spite of ligand binding intracellular signaling is blocked by tyrosine kinase inhibitors. Based on this knowledge, a clear antiproliferative effect can be predicted by both anti-EGFR strategies. However, monotherapy seemed to be less successful compared to combined treatment in achieving solid tumor control. The molecular explanation for the increased success of combination treatment with radiation, may be reasoned in the ligand-independent activation of EGFR by ionizing radiation [14]. This activation is not associated with a proliferative cell response, but seems to be more related to regulation of cell survival and DNA damage repair [14] as indicated by means of clonogenic survival assays in vitro. Both, regulation of cell survival [10] and DNA repair [55] during treatment regimens with chemo-/radiotherapy were identified as attractive molecular targets during the last years. In such a scenario it is noteworthy, that treatment with tyrosine kinase inhibitors or antibodies in combination with radiation results in inhibition of EGFR-dependent Akt phosphorylation, which is linked with regulation of cell survival [40]. Moreover, treat-

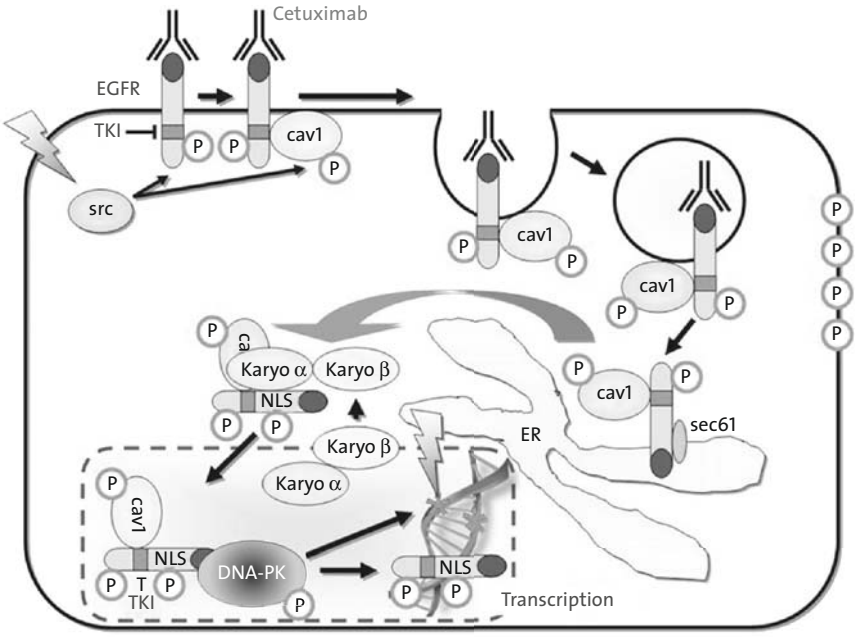

Figure 1. Role of nuclear EGFR during cellular radiation response.
Radiation activates src kinase in a so far not understood manner. Src kinase phosphorylates EGFR at residue Y845 and caveolin-1 at residue Y14, which seems to be signal for complex formation and internalization into caveolae. Incubation with cetuximab stabilizes EGFR/caveolin complexes and blocks further processing. EGFR-containing caveolae are transported into the Golgi apparatus/endoplasmatic reticulum (ER) in a microtubule-dependent way and fuse with ER membrane. EGFR is found in complex with translocon sec61 and is set free by its action into cytosol. EGFR is phosphorylated at residue T654 by means of PKCε following irradiation, which induces binding of karyopherin α and karyopherin β. This process enables transport through nuclear pore into nucleus. Karyopherins dissociate from nuclear complex and are exported back to cytosol. Nuclear EGFR either interacts with DNA-PK and is involved in activation of kinase activity essential for nonhomologous end-joining DNA repair, or acts as a transcription factor regulating expression of essential genes. There are several hints, that EGFR kinase activity is obligatory for effects of nuclear EGFR upon DNA repair. Treatment with tyrosine kinase inhibitors (TKI) may interfere with this function.

Abbildung 1. Bedeutung des nukleären EGFR während der zellulären Strahlenantwort.
Eine Bestrahlung aktiviert die src-Kinase in einer bislang unverstandenen Weise. Die src-Kinase phosphoryliert nachfolgend den EGFR am Rest Y845 und Caveolin-1 am Rest Y14. Beides sind Ereignisse, die wahrscheinlich die Komplexbildung zwischen EGFR und Caveolin-1 unterstützen und die Internalisierung des EGFR in die Caveolae auslösen. Eine Inkubation mit dem EGFR-spezifischen Antikörper Cetuximab stabilisiert den EGFR/Caveolin-1-Komplex im Zytoplasma und blockiert nachfolgende Transportprozesse. Die Caveolae mit dem EGFR werden mikrotubuliabhängig in den Golgi-Apparat/das endoplasmatische Retikulum (ER) transportiert und verschmelzen mit der ER-Membran. Der EGFR findet sich im Komplex mit dem Translocon sec61 und wird durch dessen Aktivität in das Zytoplasma freigesetzt. Nach Bestrahlung wird der EGFR am Rest T654 durch die PKCε phosphoryliert und findet sich im Komplex mit den beiden Karyopherinen α und β. Der EGFR passiert mit Hilfe dieses Transportkomplexes die Kernpore und wird in den Zellkern entlassen. Der Kerntransportkomplex löst sich auf, und die Karyopherine werden in das Zytosol zurücktransportiert. Der nukleäre EGFR beeinflusst das Zellverhalten nach Bestrahlung auf zwei Wegen. Zum einen liegt er im Komplex mit der DNA-PK vor und reguliert die Aktivität dieses für die DNA-Reparatur wichtigen Enzyms. Zum anderen wirkt der EGFR als Transkriptionsfaktor und reguliert die Transkription von proliferationsrelevanten Genen. Offensichtlich ist vor allem für die Effekte auf die DNA-Reparatur die Kinaseaktivität des EGFR im Zellkern essentiell, da sich durch den Einsatz von Tyrosinkinaseinhibitoren (TKI) die DNA-Reparaturkapazität reduzieren lässt.

ment with cetuximab can block nuclear EGFR transport in certain tumor cells, which is linked with inhibition of DNA repair [15]. However, although we observed no blockage of nuclear EGFR transport by tyrosine kinase inhibitors, a clear inhibition of DNA repair was seen [59]. This can be explained by the need of kinase activity of EGFR during regulation of DNA-PK or other nuclear proteins involved in DNA repair following irradiation. Thus the question remains unanswered, whether the anti-EGFR strategy with small molecules or antibodies is more efficient in tumor therapy. Furthermore, it is difficult to dissect the role of nuclear EGFR from "classic" membrane-associated EGFR signaling following irradiation of the cell, since both cytosolic and nuclear signaling overlay. Furthermore, it is unresolved under which molecular conditions cetuximab treatment can block nuclear EGFR transport. Further research is necessary to obtain better insights into mechanism and function of nuclear EGFR.

Conclusion

Current knowledge about nuclear transport is summarized in Figure 1. Nuclear localization of EGFR was observed either after cell stimulation with EGF or after treatment with genotoxic substances. However, the scenario described herein in fact is oversimplified, since the effects of nuclear EGFR are superimposed by the cytosolic signaling of membrane-associated EGFR. In addition, nuclear EGFR interacts with other members of the erbB receptor family also detected within the nucleus. Nevertheless, the relevance of nuclear EGFR for cell survival and DNA repair is beyond doubt. Anti-EGFR strategies, i.e., treatment with antibodies or kinase inhibitors, both can interfere with nuclear EGFR transport and function. However, the role of nuclear EGFR during tumor therapy cannot answered so far. Preclinical data demonstrate clearly, that all tumor cell lines investigated respond on irradiation with nuclear EGFR transport. Furthermore, experi-

mental knockdown of EGFR expression results in a strong radiosensitization and DNA repair is inhibited. Based on these observations, it is postulated that nuclear EGFR plays an important role during regulation of cell survival following stress exposure. However, to estimate the role of nuclear EGFR as a clinically molecular target, a selective inhibitor of nuclear EGFR transport has to be identified, which is subject of ongoing investigations.

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