Review

Interaction of EGFR with the tumour microenvironment: Implications for radiation treatment

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Abstract

Treatment failure through radioresistance of tumours is associated with activation of the epidermal growth factor receptor (EGFR). Tumour cell proliferation, DNA-repair, hypoxia and metastases-formation are four mechanisms in which EGFR signalling has an important role. In clinical trials, a correlation has been demonstrated between high EGFR expression in tumours and poor outcome after radiotherapy. Inhibition of EGFR signalling pathways improves the effectiveness of radiotherapy of head and neck cancers by overcoming these main mechanisms of radioresistance. The fact that only a minority of the patients respond to EGFR inhibitors reflects the complexity of interactions between EGFR-dependent signalling pathways and the tumour microenvironment. Furthermore, many components of the microenvironment are potential targets for therapeutic interventions. Characterisation of the interaction of EGFR signalling and the tumour microenvironment is therefore necessary to improve the effectiveness of combined modality treatment with radiotherapy and targeted agents. Here, the current status of knowledge is reviewed and directions for future research are discussed.

Tumour microenvironment

Our current conception of a malignant tumour is that of a complex structure containing not only cancer cells but also a variety of normal cell types that intimately interact with a microenvironment that is characterised by both temporal and spatial heterogeneity. It has become clear that this tumour microenvironment is important during early cancer development and progression, and is also of influence on the response of tumours to radiation [3,4]. Elements that make up the tumour microenvironment include endothelial cells of the blood and lymphatic vessels, fibroblasts, infiltrating cells of the immune system and the tumour extracellular matrix (ECM) [5]. Availability of oxygen and nutrients depends on the functionality of the vascular bed and affects the metabolic state of tumour and stromal cells. An imbalance between oxygen and glucose supply and consumption will result in hypoxia and acidification. Within the tumour microenvironment, hypoxia is relevant in almost all solid tumours. Reduced oxygen supply can be lethal for some cells, but others are able to survive under even severe or prolonged hypoxic conditions. Hypoxia-induced cell signalling promotes tumour growth, migration and survival. The development of new vasculature within a tumour involves the formation of new vessels from endothelial cells (vasculogenesis) in addition to sprouting (angiogenesis) of new vessels from existing ones [6]. New tumour blood vessels, prerequisite for tumour progression and metastasis formation, the result of interplay between pro- and anti-angiogenic factors, is
Epidermal growth factor receptor and downstream signalling pathways

EGFR is a transmembrane protein with intrinsic tyrosine kinase activity that is overexpressed in most epithelial cancers, e.g. in over 80% of head and neck squamous cell carcinomas (HNSCC) [12,13]. Overexpression could lead to resistance of tumour cells against radiation as demonstrated by in vivo studies [14–16] and is associated with poor prognosis in HNSCC [13]. (p)EGFR and HER2 expression are mostly determined by intrinsic features of the tumour cell, while the activation of downstream kinases is highly influenced by the tumour microenvironment [17]. Ligand binding to EGFR induces conformational changes leading to receptor homo- or heterodimerisation at the plasma membrane with one of its family members, ErbB2 (HER2), ErbB3 or ErbB4. This causes autophosphorylation, subsequent receptor internalisation and stimulation of multiple signalling pathways including ras-mitogen-activated protein kinase (MAPK)-extracellular signal-regulated kinase (ERK), phosphatidylinositol-3-kinase-AKT (PI3-K/AKT), signal transducers and activators of transcription (STAT) and the phospholipase C gamma (PLC-) pathways [3,18,19]. These pathways all share that they counteract radiation efficacy being involved in proliferation, migration, apoptosis and angiogenesis. Of note is that, besides through ligand binding, EGFR can also be activated by ionising radiation itself, again leading to radioresistance [18,20–22].

EGFR tyrosine kinase inhibition with erlotinib or gefitinib improves progression-free survival in advanced non-small cell lung cancer (NSCLC) with EGFR mutations [23]. In preclinical studies EGFR expression was needed for C225-response, but this was not sufficient to predict response to C225 plus radiotherapy. Evaluation of the microenvironment revealed that basal expression of additional growth factor receptors and effects on proliferation, correlated to a certain extent with response to combined C225-radiotherapy [24]. Combining radiotherapy with the blockade of EGFR by the chimeric (mouse/human) monoclonal antibody cetuximab, has resulted in improved locoregional control and survival for patients with HNSCC [25] demonstrating that EGFR is an clinically relevant target for molecular therapies in addition to radiation.

A key protein activated through EGFR is AKT that can be phosphorylated at Thr308 and Ser473 at the cell membrane after activation of PI3-K. pAKT then translocates to the cytoplasm and nucleus where it can activate or deactivate a myriad of substrates via its kinase activity or via the transcription of genes responsible for tumour progression. Although most studies correlate high pAKT expression to better survival or reduced migration [2,28], there are also studies linking high pAKT to better survival or reduced migration [2,28]. This suggests that the concept of EGFR induced AKT activation leading to treatment resistance and poor outcome is a simplification of a complex interaction between the EGFR signalling network and the tumour microenvironment. Importantly, it is shown that hypoxia can induce cellular changes and in hypoxic areas of HNSCC activated AKT has been observed in the absence of EGFR expression (Fig. 2). Immunohistochemical staining for EGFR and pAKT in biopsies of patients with HNSCC reveals a lack of association: tumour cells positive for EGFR were found negative for pAKT and vice versa (Fig. 3). Although a better correlation between activated EGFR (pEGFR) and pAKT existed, there were still tumour cells present with pAKT but no pEGFR [2]. A possible explanation is that AKT can be activated by different members of the ErbB family and other type of receptors like VEGFR. This is supported by the observation that blocking VEGFR-2 caused a suppression of pAKT [29]. Also, these observations suggest that the tumour microenvironment may stimulate the activation of AKT in an EGFR-independent manner and is likely to be involved in the other EGFR-driven signalling pathways as well.
Tumour microenvironmental factors and radioresistance

Not only do tumours from different origins respond differently to radiotherapy, also tumours with similar pathology show broad variations in response. Resistance of cancer cells to radiation is complex and many intrinsic and extrinsic factors are involved. Activation of EGFR signalling pathways can influence various cellular functions that are involved in the major mechanisms leading to radioresistance including tumour cell proliferation, DNA-repair and hypoxia. Activation of EGFR is also involved in a fourth mechanism leading to radioresistance of tumours namely the escape of tumour cells from local therapy by radiation through the formation of metastases.

Tumour cell proliferation

The accelerated proliferation rate of tumour cells during radiotherapy is one reason for locoregional failure [30]. A number of clinical trials have confirmed that shortening the overall treatment time leads to increased tumour control probability by reducing the possibility of tumour cells to enhance repopulation between radiotherapy fractions [31]. This accelerated proliferation rate can be a result of activation of EGFR in response to ionising radiation and an explanation why tumours with high EGFR expression have a worse prognosis [13]. Two clinical studies have indeed confirmed that accelerated radiotherapy, either moderate acceleration with 6 fractions per week over 5.5 weeks or accelerated hyperfractionation with 3 fractions a day, and a total treatment time of 12 consecutive days, results in better locoregional control when EGFR was overexpressed, but not in tumours with low EGFR expression [30,32]. This suggests that EGFR-related signalling is involved in the proliferative response to radiotherapy thereby enhancing tumour survival probabilities. Preclinical data confirm that radiation-induced activation of EGFR represents a critical step in the activation of this mechanism [33]. Large-scale studies with fractionated irradiation in xenografted FaDu (HNSCC) tumours demonstrated that after 3–4 weeks of fractionated radiotherapy an enhancement of repopulation occurs. At the same time an upregulation of EGFR expression was observed, indicating that EGFR is involved in this response [34]. Inhibition of EGFR through cetuximab [35] or tyrosine-kinase inhibitors such as erlotinib [15] was found to reduce tumour cell repopulation. EGFR inhibition with cetuximab during fractionated radiotherapy reduced tumour cell repopulation and improved local control in FaDu tumours [35] but also leads to a change in the spatial distribution of EGFR favouring the membranous expression [36]. Erlotinib can inhibit radiation-induced activation of EGFR thereby reducing its proliferative signalling capacity. Both agents have been demonstrated to lower the percentage of human tumour cells in the more radiosensitive S-phase fraction and induce an accumulation of cells in the more radiosensitive cell cycle phases [37]. Consistent with these findings, Krause et al. observed a significantly lower S-phase fraction measured by BrdU labelling after treatment with a tyrosine kinase inhibitor in FaDu tumours compared to the untreated tumour. However, this reduced proliferation did not lead to improved local tumour control after radiation [38]. Additionally, Gurtner et al. showed that in a panel of HNSCC models erlotinib as well as cetuximab lead to tumour growth delay but only simultaneous application of cetuximab during fractionated irradiation improved local control, while erlotinib did not enhance the radiotherapy effect [39], illustrating that different models of EGFR inhibition may lead to different ultimate treatment results.

DNA-repair

Activation of the EGFR downstream pathways RAS and PI3-K/AKT have been found to increase the resistance of tumour cells to agents that cause DNA damage [40]. DNA double stranded breaks (DSB) are the most important DNA lesions leading to cell kill after radiotherapy. Tumour cells can repair DSBs through non-homologous end-joining (NHEJ) and homologous recombination (HR). NHEJ is the major process responsible for survival of cells exposed to ionising radiation, making this type of repair probably most influential for treatment outcome. An important molecular complex involved in this process is the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), which is known to reside primarily in the nucleus. An interaction between nuclear EGFR and DNA-PKcs has been described suggesting a crucial role of nuclear EGFR for regulation of DNA repair after radiation [41]. Activated EGFR can be internalised and translocated to the nucleus and irradiation-induced nuclear EGFR can stimulate the formation of the nuclear EGFR/DNA-PKcs complex [42]. Selective inhibition of these

![Fig. 2. Immunohistochemical staining of whole consecutive laryngeal tumour sections shows that pAKT (green) expression (B) occurs predominantly in hypoxic areas stained with pimonidazole (green; A). Red is EGFR expression, mostly seen in normoxic areas closer to tumour blood vessels (white). 200× magnification. Scale bars represent 100 m.](image-url)
pathways was found effective in reducing tumour cell survival. For example, cetuximab blocked radiation-induced nuclear translocation of EGFR and was associated with the inhibition of radiation-induced activation of DNA-PKcs in a human bronchial carcinoma cell line [42]. Combined treatment of radiation and cetuximab resulted in a redistribution of DNA-PKcs from the nucleus to the cytoplasm. The reduction of DNA-PKcs in the nucleus leads to impeded NHEJ, essential for DNA-repair and survival. This might be a potential mechanism of the combined modality approach whereby the repair of DNA-DSBs after radiotherapy is impaired by cetuximab [37]. Blockage of radiation-induced DNA-PKcs activation by EGFR, PI3-K or AKT inhibition as well as through knockdown of AKT1 by siRNA indicates the requirement of the EGFR-PI3-K/AKT pathway for the regulation of DNA-DSB repair after exposure to ionising radiation [43]. Selective EGFR inhibition by gefitinib increased radiosensitivity of stem-like gliomaspheres by reducing DNA-PKcs expression, accompanied by reduced repair of radiation-induced DNA DSBs [44]. These studies suggest that EGFR mediated repair of DNA damage might play a prominent role in the mechanism of radioresistance. Further preclinical in vivo and clinical research is required to determine to what extent this is a tumour type specific mechanism and to identify predictors of response to EGFR- or downstream PI3-K/AKT-targeted DNA-repair inhibitor.

In conflict with other studies, a novel finding showed that cetuximab as well as irradiation can promote EGFR translocation to the nucleus. Cetuximab treatment resulted in phosphorylation of the EGFR Y845 site leading to an increased translocation to the nucleus of HNSCC tumour cells. This process can be inhibited by dasatinib, an inhibitor of several kinases including SRC family kinases (SKFs), which are suggested to be necessary for the phosphorylation of the EGFR Y845 site and cetuximab-induced EGFR translocation to the nucleus [45]. Whether this cetuximab-induced nuclear EGFR is able to activate target genes to the same extent as ligand binding or radiation does is still unclear.

Hypoxia

The consequences of tumour cell hypoxia for treatment and patient outcome have been well established [46,47]. Hypoxia is associated with treatment failure as hypoxic tumour cells are significantly more resistant to radiation than normoxic cells. Although hypoxia is considered a limiting factor for tumour growth it is a stimulus for invasion and metastasis formation [5]. To adapt to hypoxic conditions, cells can respond by activating hypoxia-inducible genes or pro-survival signalling pathways, directly or indirectly through induction of the transcription factor hypoxia-inducible-factor (HIF)-1 complex [48,49]. HIF-1 modulates the expression of genes involved in cell survival, angiogenesis and migration [10,50,51]. After HIF activation by hypoxia, processes are induced in cells to adapt to low oxygen levels, including metabolic changes and angiogenesis. Various treatment strategies have been developed to address the hypoxia problem [52,53]. The hypoxic sensitizer nimorazole has been shown to improve locoregional control in HNSCC when applied in conjunction with radiotherapy [54]. Also, ARCON (accelerated radiotherapy with carbogen breathing (98% O2, 2% CO2) and nicotinamide) a treatment method that aims to counteract both tumour cell repopulation and hypoxic radioresistance has demonstrated benefit for hypoxic laryngeal carcinomas [55,56]. A meta-analysis demonstrated that in level 1a there is evidence in favour of adding hypoxic modification to radiotherapy in HNSCC [53].

In biopsies of breast cancer patients, expression of HIF-1 is associated with EGFR expression [57]. A feedback loop between hypoxia-induced upregulation of HIF-1 and EGFR provides sustained signalling when oxygenation of tumour cells improves, even up to normoxic conditions. Preclinical research shows that the activation of HIF is required for the up-regulation of EGFR protein levels in hypoxic cancer cells. Conversely, EGFR-driven PI3-K/AKT activation in breast cancer cells can also lead to increased levels of HIF-1.

Fig. 3. Mismatch of EGFR (red) and pAKT (green) in tumour sections of a laryngeal carcinoma (200× magnification). A tumour section with EGFR expression is present but there are no activated AKT (white arrow) and tumour cells with EGFR-independent pAKT expression (yellow arrow). White is tumour vasculature (stained with PAL-E). Scale bar represents 100 μm.
independently of hypoxia [58]. The exact mechanism by which PI3-K/AKT signalling mediates the induction of HIF is not clear yet and is not confirmed in other tumour cell lines [59], suggesting that this phenomenon may be cell type specific. In cervix carcinoma and pheochromocytoma cells, hypoxia resulted in the induction of HIF-1 proteins with AKT activation present. However, after growth factor stimulation under normoxic conditions there was no induction of the HIF protein and/or its transcriptional activity although the treatment resulted in activation of the PI3-K/AKT pathway. These data suggest that merely the activation of this pathway may not be sufficient for the accumulation of the HIF-1 protein, at least not in all tumour types.

Thus, interactions between the EGFR-PI3-K/AKT and HIF pathways vary with tumour type and oxygenation status. Furthermore, different effects can be measured at protein and mRNA levels. For example, blocking EGFR in HNSCC cells leads to decreased translation of HIF-1 protein under hypoxia, but inhibition does not completely eliminate HIF expression under hypoxic circumstances nor are the HIF-1 mRNA levels altered [60]. Pore et al. also found that nelvavir, a drug known to inhibit PI3-K/AKT signalling, decreases HIF-1 protein expression in HNSCC and lung cancer cells indicating that hypoxia-driven EGFR signalling might act via this downstream pathway in these tumour types [61]. HIF activation can also occur via radiation-induced EGFR signalling. Recently, Lu et al. showed that cetuximab could inhibit radiation-induced HIF-1 upregulation in HNSCC [62]. This inhibitory effect of cetuximab was much weaker for hypoxia-induced HIF-1 than for radiation-induced HIF-1 expression supporting the mediator role of EGFR in the latter. In addition, a connection between EGFR and VEGF, a downstream target of HIF-1 exists and EGFR inhibition using erlotinib leads to downregulation of HIF-1 expression and decreased VEGF secretion [29]. Erlotinib improves tumour oxygenation via improved vascular perfusion but this decrease in hypoxia did not seem to have an effect in radiosensitivity in HNSCC xenografts and cells [63]. Gefitinib treatment reduced pimonidazole binding in A431 xenografts after 5 and 8 days of treatment showing that gefitinib reduces intratumoural hypoxia [64].

These data indicate that activation of hypoxia-inducible genes is cell type specific, and that there is an intricate interaction between growth factor receptor activation and microenvironmental signalling. Tumour cell hypoxia can result in creating an optimal environment for tumour regrowth by activating hypoxia-induced genes leading to angiogenesis, while it also is associated with decreased radiation-induced DNA damage and a poorer response to radiotherapy making hypoxia a key element in the clinical outcome of patients [65]. Therefore, inhibition of EGFR in combination with hypoxia modification should be further explored as it might offer a powerful strategy for treatment of a number of cancer types.

Experimental evidence has also provided a relationship between EGFR signalling and angiogenic proteins such as VEGF. Tumours often express high levels of VEGF leading to an abnormal vasculature. Vascular normalisation and an increase in tumour blood flow can be achieved by directly targeting VEGF or its receptor (VEGFR), with for example the anti-VEGFR monoclonal antibody bevacizumab. Also, EGFR inhibition with erlotinib in mice bearing SQ20B head and neck xenografts caused changes in vessel morphology, a decreased vascular permeability and an increase in tumour blood flow. This indicates that EGFR inhibition has an effect on vasculature resulting from a decrease in VEGF expression [63]. The combination of four weeks treatment with cetuximab and ZD6474, a potent inhibitor of VEGFR-2 tyrosine kinase with a significant anti-EGFR tyrosine kinase activity, showed a more significant growth inhibition in mice bearing colon or lung adenocarcinoma xenografts as compared to single agent treatment [29]. A normal tumour vasculature and improvement in tumour blood flow can lead to better drug delivery or increased tumour oxygenation, and, thereby, a better response to radiotherapy.

Metastases formation

The escape of tumour cells from the primary tumour to distant sites and subsequent formation of metastases is a fourth mechanism leading to treatment failure. During EMT, the loss of E-cadherin proteins leads to the disruption of stable adherent junctions. In addition, cells develop a more mesenchymal phenotype, e.g. increased expression of mesenchymal proteins like vimentin, and thereby more able to spread to secondary locations in the body. Increasing evidence indicates that EGFR signalling pathways are implicated in the regulation of proteins involved in EMT. In cervical as well as prostate cancer cells lines, EGF treatment significantly decreased the abundance of E-cadherin protein and upregulated vimentin expression [66,67]. Also, in surgical biopsies of cervical carcinomas EGFR overexpression was accompanied by decreased E-cadherin and increased vimentin expression seen by immunofluorescent staining [66].

More evidence was provided by studies using EGFR inhibitors. An HNSCC tumour model in which cells dominantly express epithelial markers was found to be very sensitive to cetuximab, whereas those expressing mesenchymal markers revealed low sensitivity [68]. Although the exact interaction was not elucidated, this response to EGFR inhibition suggests that there is an association between EGFR pathways and EMT. However, this interaction between EGFR signalling and EMT seems to be tumour line specific. Combining cetuximab with irradiation induced EMT in the cetuximab-sensitive cells while triggering the reverse mesenchymal-epithelial transition (MET) in the more mesenchymal cell line [68]. The idea is that cells that have undergone EMT become less dependent on EGFR signalling for cell proliferation and survival and are thus less responsive to EGFR inhibitors [69]. This also points towards a possibility of using EMT-related proteins as predictive markers for sensitivity to cetuximab. This hypothesis needs to be confirmed in tumours from patients either sensitive or resistant to EGFR inhibition but it clearly suggests a potential for individualised treatment approaches employing EGFR-targeting or more aggressive anti-metastasis treatment based on EMT phenotype.

Conversely, it has been shown that E-cadherin-mediated-cell–cell adhesion can trigger a ligand-independent activation of EGFR thereby regulating various signalling pathways such as MAPK and AKT [70,71]. Reddy et al. showed that the activation of AKT and MAPK by E-cadherin mediated cell–cell adhesion in ovarian cancer cells is regulated by EGFR activation. However, no direct physical interaction between E-cadherin and EGFR could be detected in the cells used, suggesting the existence of intermediate molecules [71]. In contrast, it was demonstrated that in oral squamous carcinoma cells E-cadherin can physically interact with and activate EGFR, leading to the activation of MAPK [70]. This interaction leads to EGFR-E-cadherin complex formation at cell–cell junctions and receptor oligomerisation. Although the exact mechanism is unclear and needs to be verified, both studies show that E-cadherin may not only act as an adhesion molecule but also as an upstream regulator that triggers EGFR signalling pathways.

Further, a relation between oxygenation status and EMT also exists. Hypoxia can down regulate E-cadherin and upregulate mesenchymal markers, indicating that hypoxia can contribute to metastases-formation through the induction of EMT [72]. The morphological transformation induced by hypoxia in breast cancer and FaDu cells associated with EMT can be reversed after re-oxygenation or by repression of HIF-1 [72,73]. This finding at least partly explains the relation between tumour cell hypoxia, migration and ultimately metastasis formation.
Conclusions

There is strong evidence, both from preclinical and clinical studies that there is a positive correlation between the levels of EGFR found in tumour cells and resistance to radiation therapy and consequently treatment failure. EGFR signalling pathways are implicated in all major mechanisms of radioresistance. The tumour microenvironment has important influences on EGFR signalling. The fact that only a minority of the patients respond to EGFR inhibitor reflects the complexity of interactions between the EGFR-dependent signalling pathways and the tumour microenvironment. To improve the effectiveness of combined modality treatment with radiotherapy and targeted agents two strategies should be explored. One is patient selection based, not only on EGFR expression patterns, but also on microenvironmental characteristics to identify the tumour phenotypes that are most likely to benefit from the combined approach. Second is to combine radiotherapy, not only with EGFR signalling inhibition but also with treatments that counteract microenvironmental resistance mechanisms such as hypoxia, e.g. nitromazole, ARCON or angiogenesis inhibition (VEGF inhibitors). Preclinical and clinical studies should focus on these multimodality approaches with mechanistic basis to bring cancer research forward.

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References


