Subcellular localization of EGFR in esophageal carcinoma cell lines

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Abstract Background: The EGF receptor is a therapeutic target in cancer cells, whereby mutations of EGFR and/or signaling members act as predictive markers. EGFR however also exhibits dynamic changes of subcellular localization, leading to STAT5 complex formation, nuclear translocation and induction of Aurora-A expression in squamous cancer cells. We previously described high EGFR and Aurora-A expression in esophageal cancer cells. Here, we investigated subcellular localization of EGFR and STAT5 in esophageal cancer cells. Results: Quantitative immunofluorescence analyses of four esophageal cancer cell lines reflecting esophageal squamous cell carcinomas (ESCC) and esophageal adenocarcinomas (EAC) revealed that the subcellular localization of EGFR was shifted from a membranous to cytoplasmic localization upon EGF-stimulation in OE21 (ESCC) cells. Thereby, EGFR in part co-localized with E-Cadherin. In parallel, phosphorylated STAT5-Tyr694 appeared to increase in the nucleus and to decrease at the cell membrane. In three additional cell lines, EGFR was only marginally (Kyse-410/ESCC; OE19/EAC) and weakly (OE33, EAC) detectable at the cell membrane. Partial co-localization of EGFR and E-Cadherin occurred in OE33 cells. Post EGF-stimulation, EGFR was detected in the cytoplasm, resembling endosomal compartments. Furthermore, OE19 and OE33 exhibited nuclear

STAT5-Tyr694 phosphorylation upon EGF-stimulation. None of the four cell lines showed nuclear EGFR expression and localization. Conclusion: In contrast to other (squamous) cancer cells, activation of EGFR in esophageal squamous cancer cells does not result in nuclear translocation of EGFR. Still, the subcellular localization of EGFR may influence STAT5-associated signaling pathways in esophageal cancer cells and hence possibly also the responses to ErbB, respective EGFR-targeted therapies.

Keywords Esophageal cancer cells · EGFR · STAT5 · Subcellular localization

Abbreviations

EAC	Esophageal Adenocarcinoma
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ESCC	Esophageal Squamous Cell Carcinoma
PBS	Phosphate buffered saline
STAT	Signal Transducer and Activator of Transcription

Introduction

Among the most common cancer types, esophageal cancer is still one of the deadliest, mainly because of the late onset of specific symptoms (Enzinger and Mayer 2003). Esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC) constitute about 90 % of all histological subtypes. Both are associated with different risk factors, mainly local irritation and Barrett's esophagus for EACs (Enzinger and Mayer 2003).

Epidermal growth factor receptor (EGFR) belongs to the EGFR family of receptor tyrosine kinases. Its eponymous

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ligand is the epidermal growth factor (EGF). Upon ligand binding, EGFR undergoes dimerization and autophosphorylation followed by activation of multiple signalling pathways, including the signal transducer and activation of transcription (STAT) pathway (Linggi and Carpenter 2006). EGFR gene amplification and/or overexpression occur in various cancers including ESCCs and EACs and are associated with worse prognosis and treatment response to EGFR-targeted therapies (Aichler et al. 2014; Cronin et al. 2011; Kato et al. 2013; Ku and Ilson 2013; Lorenzen et al. 2015; Marx et al. 2010). In esophageal cancers, specific EGFR expression and dimerization patterns guide cancer cell survival, cell migration and EGFR-targeted therapy responses (Fichter et al. 2014b; Fichter et al. 2014a).

Like other receptors, EGFR can shift between different cell compartments. Activated EGFR may internalize via Clathrin-dependent or Clathrin-independent endocytosis (Wang et al. 2010). From the early endosomes, EGFR may shuttle back to the cell surface membrane, to lysosomes for degradation or to other compartments, including the nucleus (Wang and Hung 2012). Nuclear EGFR was associated with cell proliferation, inflammation, DNA repair as well as resistance to chemo- and radiotherapy either via transcriptional regulation or via local signal transduction (Wang and Hung 2009).

STAT proteins are transcription factors activated by phosphorylation through EGFR directly or via EGFR-activated tyrosine kinases like Src or Janus kinase (Mirmohammadsadegh et al. 2006; Quesnelle et al. 2007). There are seven STAT proteins, 1–4, 5A/B and 6 that have partially cancer-promoting potential. STAT5 (A and B) generally promotes cell proliferation (Alvarez and Frank 2004). In addition, STAT5A may complex with EGFR, translocate to the nucleus and bind to the Aurora-A promoter in an EGFR-overexpressing squamous-cell carcinoma cell line, thereby also causing chromosomal instability (Hung et al. 2008).

Recently, we demonstrated that Aurora-A plays an important role in esophageal cancer cells (Fichter et al. 2011) and that EGFR and STAT3 are overexpressed and activated preferentially in ESCCs (Fichter et al. 2014b; Fichter et al. 2014a; Timme et al. 2013). Moreover, we previously showed that in ESCC cells (cell line OE21) EGF stimulation activated EGFR signalling and induced STAT3 phosphorylation, whereas this was not the case for EAC cells (cell line OE33) (Timme et al. 2013).

However, so far little is known about the role of EGFR and STAT5 in potentially mediating deregulated Aurora-A expression in these esophageal cancer cells, especially in ESCC OE21 cells showing prominent EGFR overexpression (Fichter et al. 2014b). Therefore, the aim of the present study was to examine EGFR activation, subcellular localization and association with STAT5 for nuclear translocation.

Materials and methods

Cell culture

Esophageal squamous cell carcinoma (OE21, KYSE-410) and adenocarcinoma (OE19, OE33) cell lines (European Collection of Cell Cultures of the Health Protection Agency, Salisbury, UK) were cultivated in RPMI 1640 with 2 mM L-Glutamine and 10 % FCS (PAA Laboratories) at 37 °C with 5 % CO₂. All cell lines were characterized by us before (Ahrens et al. 2015; Fichter et al. 2011; Fichter et al. 2014a; Fichter et al. 2014b; Timme et al. 2013).

For EGF-stimulation, cells were cultivated in a culture dish for up to three days, washed in 1xPBS and starved for 24 h in RPMI 1640 with 2 mM L-Glutamine medium without FCS. Then, 100 ng/ml EGF was added for 30 min prior to subsequent analyses.

Immunofluorescence staining

Cells were fixed with formaldehyde (2 % aq, 20 min) and permeabilized with methanol (10 min, −20 °C). After blocking binding sites with goat serum in PBS for 1 h, cells were incubated with the primary antibody overnight. The secondary antibody was applied for 1 h. For DNA staining, Vectashield® Mounting Medium with DAPI (Vector Laboratories) was used. Primary antibodies were: mouse monoclonal Anti-Human EGFR, clone H11 Core M3563 IgG1 (1:500; DakoCytomation, Glostrup, Denmark), rabbit monoclonal antibody Phospho-Stat5 (Tyr 694) clone C11C5 (1:50; Cell Signaling Technology), and as negative control a ChromPure Mouse/Rabbit IgG, whole molecule (1:500 for negative control of EGFR and 1:50 for negative control of P-STAT5 stainings; Jackson ImmunoResearch Laboratories). Secondary antibodies were: Alexa Fluor® 488 goat anti-mouse or Alexa Fluor® 568 goat anti-rabbit IgG (H + L) (1:200; Life Technologies).

Stainings of three independent experiments were examined using a ZEISS Axioplan 2 imaging microscope, an ApoTome cross section module and Axiovision 4.8 software (Carl Zeiss Microscopy). Per cell line and experiment, images were taken using a 63× objective at three random positions, each showing up to 63 cells. For each cell line, the predominant subcellular localization (membranous, cytoplasmic, nuclear) of EGFR and P-STAT5 was determined and quantified in each three images from each three independent experiments. Images were analyzed in 3D and are shown in Figures as 2D slices for optimal visualization of the membranous and nuclear expression. The frequencies of the predominant staining patterns observed in each cell line of the three experiments are given as percent (arithmetic mean weighted by the number of images ± weighted standard deviation).

To analyze EGFR membranous expression in relation to E-Cadherin, immunofluorescence double-stainings were performed in all four cell lines as above, using a rabbit monoclonal anti-E-Cadherin antibody (clone 24E10, Cell Signalling Technologies). Images were taken using a 63 $\times$ objective at random positions. Images of EGFR and E-Cadherin double immunofluorescence are provided as 2D slices of the 3D image stack.

RNA isolation, cDNA synthesis and qRT-PCR

Total RNA isolation (RNeasy[®] Mini kit; Qiagen, Hilden, Germany), reverse transcription, and qRT-PCR were performed for Cyclin-D1, Aurora-A, Aurora-B, Bcl-x1, Survivin, STAT1, STAT3, STAT5 and TBP (reference) as established before (Fichter et al. 2011; Timme et al. 2013). Data reflects “relative mRNA expression” (y-axes in graphs) of independent experiments (mean $\pm$ standard deviation).

Western blot

Total protein extracts were obtained using the Qproteome[®] Mammalian Protein Prep Kit (Qiagen) following the manufacturer's protocol (Fichter et al. 2014a; Fichter et al. 2014b; Timme et al. 2013). The extracts' protein concentrations were measured using the DC[™] Protein Assay (Bio-Rad Laboratories).

Per lane, 5 μ g total protein was loaded on a 10 % polyacrylamide gel. After SDS-gel electrophoresis, the proteins were transferred onto a Protran[®] Nitrocellulose Transfer Membrane (Whatman) using a wet blot protocol. 5 % nonfat dried milk powder in TRIS buffered saline was used for blocking. The primary antibody was applied overnight, the secondary antibodies for 1 h with multiple intermediate TBS/T and TBS washing steps. Lumigen[™] TMA-6 Solution (Lumigen) and Amersham[™] Hyperfilm[™] ECL (GE Healthcare) were used for chemiluminescence. Primary antibodies against EGFR and P-STAT5 equaled those used for immunofluorescence stainings, with dilution 1:1000. The secondary antibodies were: Peroxidase-conjugated AffiniPure Goat Anti Mouse or Goat AntiRabbit IgG (H + L) (1:25,000; Jackson ImmunoResearch Laboratories).

Results

Subcellular localization of EGFR and P-STAT5 is differentially influenced by EGF-stimulation in esophageal cancer cells

In view of the reported EGFR and STAT5 complex formation and nuclear translocation (Wang et al. 2010) (Wang and Hung

2009) (Hung et al. 2008), the subcellular localization of EGFR and P-STAT5 were investigated in four esophageal carcinoma cell lines using double immunofluorescence staining. Figure 1 shows representative images and Fig. 2 shows the quantification.

First, unstimulated OE21 cells exhibited the most intensive expression of EGFR, whilst Kyse-410, OE19 and OE33 cells showed only marginal EGFR expression (Fig. 1, left column). In addition, 87 % of unstimulated OE21 cells showed membranous EGFR localization (Fig. 1, left panels; Fig. 2a), whereas only a small fraction of the other cells expressed EGFR at the membrane (Kyse-410: 9 %, OE19: 2 %, OE33: 33 %). Weak EGFR expression in these cell lines was instead mainly found in the cytoplasm (Kyse-410: 11 %, OE19: 91 %; OE33: 57 %; Fig. 1, left panels; Fig. 2b and d), with few

Fig. 1 Subcellular localization of EGFR and P-STAT5 in esophageal carcinoma cells. The figure shows representative images of double immunofluorescence stainings (EGFR: green, P-STAT5: red, DNA: blue) of unstimulated (left) and EGF-stimulated (100 ng/ml for 30 min, right) esophageal cancer cell lines. Note membranous localization of both EGFR and, in part P-STAT5 (arrow), in mainly OE21 cells. Note nuclear P-STAT5 expression in OE21, but also in OE19 cells (arrowheads). Cytoplasmic P-STAT5 is expressed in all cell lines. See Fig. 2 for quantification. Scale bar =20 μ m

Fig. 2 Quantitative analysis of subcellular localization of EGFR and P-STAT5. The graphs show the quantification of double immunofluorescence analyses of EGFR (a-d) and P-STAT5 (e-h) protein expression and localization in three independent experiments (see Fig. 1 and Materials and

Methods for details). The vertical axis defines the percentage of cells with the respective dominating subcellular staining pattern. (a, e) OE21, (b, f) Kyse-410, (c, g) OE19 and (d, h) OE33 cells

membranous EGFR signals in OE33 cells (Fig. 1, lower left panel; Fig. 2d).

Upon EGF-stimulation, EGFR shifted from the membrane to the cytoplasm in OE21 cells, with 86 % of OE21 cells showing cytoplasmic EGFR (Fig. 1, top right panel; Fig. 2a). Thereby, the EGFR staining pattern resembled those of the endosomal compartment, characterized by cytoplasmic, non-confluent round vesicles. In contrast, EGF-stimulation of Kyse-410, OE19 and OE33 cells only resulted in a moderate shift of EGFR to the cytoplasm in Kyse-410 (unstimulated: 11 %; EGF-stimulated: 33 %) and OE33 (unstimulated: 57 %, EGF-stimulated: 94 %), whereas unstimulated (91 %) and EGF-stimulated (96 %) OE19 cells did not show a prominent shift of EGFR subcellular localization (Fig. 1 right panels; Fig. 2b and d).

Second, phosphorylated STAT5 was detected in all subcellular localizations, however with distinct patterns. In unstimulated OE21 cells, 47 % showed primarily nuclear, 30 % membranous and 19 % cytoplasmic P-STAT5 staining (Fig. 1 left panels, Fig. 2e). A similar distribution was seen for unstimulated Kyse-410 and OE33 cells, although both were exhibiting a more predominant cytoplasmic localization (Kyse-410: 64 %; OE33: 43 %; Fig. 1 left panels, Fig. 2f and h). In contrast, 58 % of unstimulated OE19 cells showed P-STAT5 primarily within the nucleus (Fig. 1 left panels; Fig. 2g).

Upon EGF-stimulation, P-STAT5 underwent nuclear translocation in OE21, OE19 and OE33 cells, but not in KYSE-410 cells (Fig. 1, right panels; Fig. 2e and h). Whereas in OE21 and OE33 cells there was a concomitant loss of membranous P-STAT5 localization (Fig. 2e and h), this did not occur for OE19 cells (Fig. 2g) which showed loss of cytoplasmic P-STAT5.

Finally, only OE21 cells exhibited a partial co-localization of EGFR and P-STAT5 at the cell membrane, but neither in the cytoplasm nor in the nucleus (Fig. 1, top panels).

These data indicate that there is an EGF-dependent association of EGFR and P-STAT5 in particularly OE21 cells.

EGFR co-localizes with E-cadherin in OE21 cells

To further analyze the distinct patterns of EGFR subcellular localization in correlation to E-Cadherin, double immunofluorescence stainings of EGFR and E-Cadherin were performed in the four cell lines.

In unstimulated OE21 cells, strong EGFR expression partially showed co-localization with E-Cadherin at the cell membrane. Upon EGF-stimulation, this EGFR and E-Cadherin co-localization was also observed in the cytoplasm resembling endosomal compartments (Fig. 3).

In unstimulated Kyse-410, OE19 and OE33 cells, E-Cadherin expression was more prominent than EGFR expression and strongly visible at the cell membranes. This did not change upon EGF-stimulation. In contrast, EGFR was again found in the cytoplasm of Kyse-410 and OE33 cells upon EGF-stimulation, but not in complex with E-Cadherin as seen for OE21 cells.

Effects of EGFR activation and localization on STAT5 phosphorylation and gene expression patterns

To further validate EGFR expression and STAT5 phosphorylation upon EGF-stimulation, Western blot analysis was performed in unstimulated and EGF-stimulated (30 min) cells (Fig. 4a). As expected, OE21 (ESCC) cells expressed

Fig. 3 Subcellular localization of EGFR and E-Cadherin protein expression in esophageal cancer cells. The figure shows representative images of double immunofluorescence stainings (EGFR: green, E-Cadherin: red, DNA: blue) of unstimulated (left) and EGF-stimulated (100 ng/ml for 30 min, right) esophageal cancer cell lines. Note co-localization (yellow) of EGFR and E-Cadherin in unstimulated and EGF-stimulated OE21 cells as well as in unstimulated OE33 cells. Note that EGFR and E-Cadherin are transferred to the cytoplasm in OE21 cells upon EGF-stimulation (white arrow). Note that E-Cadherin expression is generally weaker in OE21 as compared to Kyse-410, OE19 and OE33 cells. Scale bar =50 µm

EGFR, which was increased by EGF-stimulation (Fig. 4a). In contrast, neither Kyse-410 (ESCC), nor OE19 (EAC), nor OE33 (EAC) cells expressed detectable EGFR levels, irrespectively of EGF-stimulation.

To examine whether the changes of subcellular localization of EGFR and STAT5A under EGF stimulation alter gene expression, q-RT-PCR was performed for Aurora-A (Hung et al. 2008; Wang and Hung 2009; Wang et al. 2010) and other selected genes involved in cell proliferation and survival (Timme et al. 2013) in OE21 cells exhibiting high EGFR expression and a shift of EGFR subcellular localization post EGF stimulation. As shown in Fig. 4b, there was no significant change of Aurora-A or Aurora-B, Cyclin-D1, Bcl-xl and Survivin as well as STAT1, STAT3 or STAT5 mRNA expression upon EGF stimulation as compared to unstimulated cells.

Discussion

Whilst the main signaling function of EGFR is linked to its membranous localization (Wang and Hung 2012), nuclear EGFR may function as transcription factor mediating growth promotion or therapy resistance (Wang and Hung 2009). For example, in epidermoid squamous cancer cells, STAT5 interacts with EGFR in activating gene expression of Aurora-A (Hung et al. 2008), a kinase also closely associated with chromosomal instability of esophageal cancer (Fichter et al. 2011). Indeed, studies indicate a negative prognostic effect of nuclear EGFR in breast cancer (Hadzisejdić et al. 2010), ovarian cancer (Xia et al. 2009) and esophageal squamous cell carcinoma (Hoshino et al. 2007).

Therefore, this study addressed EGFR and P-STAT5 subcellular localization in four esophageal cancer cell lines, which differentially express EGFR (Fichter et al. 2014a; Fichter et al. 2014b) and Aurora kinases (Fichter et al. 2011).

Indeed, EGFR and P-STAT5 expression was found in all four esophageal cancer cell lines, but only the ESCC cell line OE21 exhibited strong membranous EGFR expression, which co-localized partially with E-Cadherin and weakly also with P-STAT5. In contrast, the other ESCC cell line Kyse-410 or the EAC cell line OE19 showed marked membranous E-Cadherin expression without concomitant EGFR expression. In the EAC cell line OE33, weaker membranous EGFR expression also partially co-localized with E-Cadherin expression. Nuclear localization of EGFR with P-STAT5 as complex was undetectable in all four esophageal cancer cell lines. This was also unaffected by EGF-stimulation of the cells. Still, a weak nuclear shift of only P-STAT5 was observed in the analyzed cell lines, except Kyse-410.

Together, this is in accordance with our previous study in human tissue specimens of esophageal cancers, which did not reveal any nuclear EGFR expression (Fichter et al. 2014b). Still, another study observed nuclear EGFR in esophageal carcinomas, which correlated with poor prognosis (Hoshino et al. 2007). However, in these two studies different methodologies and patient cohorts were used for analyses. The previously reported nuclear EGFR/P-STAT5 pathway including subsequent Aurora-A transactivation in squamous cancer cells (Hung et al. 2008) therefore does not represent a prominent signaling pathway in (squamous) esophageal cancer cells in situ or in vitro. This is also reflected by our here presented experiments showing unaltered patterns of Aurora-A, Aurora-B, Cyclin-D1, Bcl-xl and Survivin as well as STAT1, STAT3 and STAT5 mRNA expression upon EGF-stimulation. To rule out that mRNA expression of these candidate genes was unaffected due to inappropriate EGFR signalling pathway activation, we showed that EGF-stimulation clearly induces STAT5 phosphorylation (this study) and STAT3

Fig. 4 Effect of EGF stimulation on EGFR and phospho-STAT5 expression and down-stream mRNA expression patterns of OE21 esophageal cancer cells. **a** EGFR and P-STAT5 expression in ESCC and EAC cells. Western blots of total protein lysates without and with 100 ng/ml EGF-stimulation for 30 min. α -Tubulin was used as loading control. Note marked up-regulation of P-STAT5 levels in OE21 cells upon EGF-stimulation. Note lack of EGFR, but EGF induced moderate

up-regulation of P-STAT5 in Kyse-410, OE19 and OE33 cells. **b** The graph shows the fold change of mRNA expression of the selected genes in EGF-stimulated relative to unstimulated OE21 cells. Note that EGF-stimulation does not significantly alter mRNA expression of the selected genes, which remains close to “1” (normalized to unstimulated cells, unstimulated cell set to “1”)

phosphorylation (Timme et al. 2013) in Western blot analyses of OE21 cells.

Irrespective of the above, the subcellular localization of EGFR and P-STAT5 in “resting” and EGF-stimulated esophageal cancer cell lines were demonstrated here for the first time. The strongly EGFR expressing ESCC cell line OE21 clearly exhibits a distinct pattern of EGFR recycling and internalization from the cell membrane and EGFR signaling pathway activation by EGF. Thereby, EGFR partially co-localized with E-Cadherin, suggesting caveolin activation and endocytosis. In contrast, other ESCC (Kyse-410) and EAC (OE19) cell lines only marginally express EGFR and hence are largely unresponsive to ligand stimulation. In the EAC cell line OE33, EGFR expression is found at the cell membrane and this also in part co-localizes with E-Cadherin. Upon EGF-stimulation, EGFR also shuttles to the cytoplasm in OE33 cells. This is in agreement with our previous data, showing specific EGFR-HER2 heterodimer associated signaling in this cell line (Fichter et al. 2014a; Fichter et al. 2014b).

Moreover, the OE21, OE19 and OE33 cell lines showed altered P-STAT5 localization upon EGF stimulation. Thus, EGF may have acted on other receptors or EGFR

heterodimers, which undergo faster membrane recycling to endosomes not detectable by standard immunofluorescence. Interestingly, membranous and intracellular EGFR have different substrate specificities, suggesting that endocytosed EGF and “signaling endosomes” may act in cells lacking stable membranous EGFR (Burke et al. 2001; Vieira et al. 1996). Whether or not such endosomal EGFR function could modulate responses of cancer cells to EGFR inhibitors, still needs to be shown.

Together, our present study demonstrates detailed subcellular localization of EGFR and P-STAT5 in four different esophageal cancer cell lines and that activation of EGFR in esophageal squamous cancer cells does not result in STAT5 complex formation, nuclear translocation and Aurora-A expression. Still, the study suggests that subcellular localization of EGFR in the distinct histotypes of esophageal cancer may influence STAT5-associated signaling pathways and possibly therapeutic responses to evolving targeted therapies in esophageal cancer cells.

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Authors contributions LS, CF: Performed and analyzed experiments, wrote manuscript; MW: contributed morpho-/cytological interpretation, discussed results, approved manuscript, provided resources; SL: Designed study, analyzed data, wrote manuscript, provided resources.

Compliance with ethical standards

Competing interests The author(s) declare that they have no competing interests.

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