Activation of epidermal growth factor receptor results in Snail protein but not mRNA overexpression in endometrial cancer

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Abstract

Reduced E-cadherin expression is associated with tumour progression of many carcinomas, including endometrial cancers. The transcription factor Snail is known as one of the most prominent transcriptional E-cadherin repressors; its regulation in cancer tissues, however, still remains unclear. Here, we report that activation of epidermal growth factor receptor (EGFR) resulted in overexpression of Snail and also identified critical downstream signalling molecules. Stimulation of two endometrial carcinoma cell lines with epidermal growth factor (EGF) lead to an increase of Snail protein expression. In primary human endometrioid endometrial carcinomas Snail protein expression correlated with the activated, phosphorylated form of EGFR (Tyr1086) as revealed by profiling 24 different signalling proteins using protein lysate microarrays. In addition, we observed an inverse correlation between Snail and E-cadherin protein levels in these tumours. Most likely, p38 MAPK, PAK1, AKT, ERK1/2 and GSK-3β are involved in the up-regulation of Snail downstream of EGFR. Snail mRNA expression did not show a correlation with activated EGFR in these tumours. Taken together, profiling of signalling proteins in primary human tissues provided strong evidence that EGFR signalling is involved in Snail protein overexpression.

Keywords: EGFR • Snail • E-cadherin • protein microarray • endometrial carcinoma • Her2

Introduction

In developed countries, endometrial cancer is the most common cancer of the female genital tract and in North America the eighth commonest cause of death from cancer in the female population [1–3]. Around 80% of all endometrial carcinomas are of the endometrioid type [2]. Previous studies indicated that reduced expression of the calcium-dependent cell adhesion molecule E-cadherin is associated with metastases in endometrial cancers [4, 5].

Reduction of membranous E-cadherin expression is modified by various mechanisms like gene mutations [6–8], promoter hypermethylation [8–10], post-translational modification [11] and transcriptional repression [12–14]. Snail is one of the most prominent transcriptional repressors of E-cadherin. It binds to the E-boxes of the E-cadherin promoter and represses gene transcription [12, 13]. Snail is regulated on the transcriptional level as well as on the protein level. The Snail protein is highly unstable (half-life about 25 min.) [15]. Glycogen synthase kinase-3β (GSK-3β) and p21-activated kinase (PAK1) [16] were reported to control Snail's sub-cellular localization and to regulate Snail protein stability [15, 17]. Previous studies showed that overexpression of Snail in endometrial carcinoma metastases correlated to a higher tumour grade and to abnormal E-cadherin expression [18, 19].

Abnormal epidermal growth factor receptor (EGFR) signalling is an important cause of tumour progression and metastasis [20]. Epidermal growth factor (EGF) is a critical regulatory component

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of cell growth and differentiation [21, 22]. Autophosphorylation of activated EGF receptors stimulates several signal transduction pathways, including the MAP (mitogen-activated protein) kinase pathway [21]. Chronic treatment with EGF leads to down-regulation of E-cadherin expression on the transcriptional level and loss of cell–cell adherence junctions [23].

It has been reported that EGFR activates the human TWIST gene expression *via* binding of STAT3 (Signal transducers and activators of transcription 3) to the human TWIST promoter [20] and that EGF-induced transcriptional up-regulation of Snail is necessary for down-regulation of E-cadherin in 293T cells [23], a variant of human embryonic kidney cells that contain the SV40 large T antigen.

Recent reviews [24] reported that abnormal receptor tyrosine kinase signalling plays a role in the regulation of epithelial-mesenchymal-transition (EMT) during development and in cell culture studies. However, studies demonstrating a clear correlation between receptor tyrosine kinase signalling and Snail, a master regulator of EMT, in primary cancers are still missing. Given the association between EGFR overexpression and high metastatic potential, we suggested that the EGFR pathway may contribute to the regulation of Snail expression in human cancers, focusing on endometrial tumours. Here we report that activation of EGFR correlates with Snail protein overexpression and simultaneous down-regulation of E-cadherin in human endometrial carcinomas and determined down-stream signalling proteins involved in these processes.

Material and methods

Cell culture conditions

The human endometrial adenocarcinoma cell lines Ishikawa (ECACC No. 99040201) and Ishikawa (Heraklio) 02 ER- (ECACC No. 98032302) were cultured in Dulbecco's Modified Eagle Medium (Invitrogen, Paisley, UK) supplemented with 10% foetal calf serum (PAA, Coelbe, Germany) at 37°C and 5% CO_2 in a fully humidified atmosphere.

Protein kinase inhibitors and EGF stimulation

The protein kinase inhibitors were obtained from Calbiochem/MerckKGA (Darmstadt, Germany). AG1478, SB203580, UO126 were used at a concentration of 10 μM, LY294002 was used at a concentration of 20 μM. Cells were serum-starved for 24 hrs and incubated for 60 min. with inhibitors prior to stimulation with EGF (Calbiochem/MerckKGA, Darmstadt, Germany) used at a concentration of 100 ng/ml.

Tissue samples

Seventeen formalin-fixed and paraffin-embedded (FFPE) primary endometrial carcinomas (endometrioid subtype, World Health Organisation), diagnosed between 1991 and 2003 were selected from the archive of the Institute of Pathology, Technical University of Munich, Germany (Table S1). The study was approved by the Ethics Committee of the Technical University of Munich. Grading was defined according to the recommendations of the World Health Organisation.

Reference haematoxylin/eosin stained sections of the tissues were histological verified by an experienced pathologist (AW). Only tissues with at least 85% of tumour cells were included in the study. Different areas within each tumour were sampled. Adjacent unstained sections of the same paraffin blocks were used for RNA and protein extraction by microdissection as described below.

RNA extraction from cultured cells

RNA was purified by Trizol® (Invitrogen, Karlsruhe, Germany), followed by precipitation with isopropanol. The RNA pellet was washed twice in 70% ethanol, air dried and resuspended in RNase-free water.

RNA extraction from FFPE tissues

RNA was extracted as previously described [25, 26].

Agilent microarrays

Cy3-labelled cRNA was generated from 1 ug total RNA using the Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies, Böblingen, Germany). The labelled cRNA was purified with RNeasy mini columns (Qiagen, Hilden, Germany). The cRNA concentration, Cy3 incorporation and purity were determined spectrophotometrically using a Nanodrop ND 1000 spectrophotometer (NanoDrop Technologies, Thermo Scientific Nanodrop products, Wilmington, DE, USA) and the length of the amplified product determined using the Agilent Bioanalyzer. Equal amounts (1.65 μg) of purified cyanine 3-labelled cRNA were hybridized for 17 hrs at 65°C with the Agilent whole human genome microarrays (4 × 44K) in an Agilent hybridization oven rotating at 10 rpm. The arrays were washed and processed with Agilent Washing buffers and Agilent Stabilisation and Drying Solution.

The microarrays were scanned at a resolution of 5 μm on an Agilent scanner using extended dynamic range (PMT 10/100). The image data were processed using default values in Feature Extraction version 9.5. The expression data were loaded into GeneSpring 7.2 to perform median normalization, filtering and t-tests.

Reverse transcription

RNA from cell lines and tissues was reverse-transcribed in a final volume of 20 μl using Superscript II reverse transcriptase (Invitrogen, Karlsruhe, Germany) according to manufacturer's instructions with the following conditions: 10 mmol/l dNTPs (Roche Diagnostics GmbH, Mannheim, Germany), 2 U/μl RNase OUT ™ (Invitrogen, Karlsruhe, Germany), 0.15 μg/ml random hexamers (Invitrogen, Karlsruhe, Germany) and 1 μg RNA. The reaction was performed at 42°C for 60 min., followed by inactivation of the enzyme at 70°C for 15 min.

Quantitative real-time PCR

The primers and probes were designed to span an intron to exclude annealing to genomic DNA as previously described [25]. Relative amounts of mRNA were calculated by the standard curve method in relation to Glyceraldehyd 3-phosphate dehydrogenase (GAPDH) levels. The TaqMan® Runs were done in duplicate and repeated in three independent experiments (only for cell culture experiments).

Preparation of protein lysates from cultured cells

Cells were washed in PBS and lysed in T-Per buffer (PIERCE, Rockford, IL, USA) supplemented with 50 mM Sodium-fluoride, 1 mM Sodium-orthovanadate, 10 mM β-Glycerophosphate, 1 mM Phenylmethylsulfonylfluoride and 1 complete mini protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany) per 10 ml lysis buffer. Protein concentration was assessed by the Bio-Rad assay method (Bio-Rad Laboratories, Munich, Germany).

Protein extraction from formalin-fixed tissue samples

After standard deparaffination of tissue sections (10 μm), proteins were extracted as previously described [27], using the Qproteome FFPE Tissue Kit (Qiagen).

Immunoblotting

Total protein extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotting was performed on nitrocellulose membranes (Whatman/Schleicher and Schuell, Dassel, Germany). Blots were developed using the ECLPlus and ECLadvance Western Blot Detection System (Amersham/GE Healthcare Europe GmbH, München, Germany).

Protein lysate microarrays

Protein lysate microarrays (also called reverse phase protein microarrays) were generated using a hand-held spotter ('MicroCaster') according to the instructions of the manufacturer (Whatman/Schleicher and Schuell, Dassel, Germany). For every lysate and every dilution (undiluted, 1:2, 1:4, buffer), 4 replicates were applied onto a nitrocellulose coated glass slide (Fast™Slide, Whatman/Schleicher and Schuell, Dassel, Germany). Lysate microarrays were developed using the ECLPlus and ECLadvance Western Blot Detection System. For estimation of total protein amounts, parallel arrays were stained with Sypro Ruby Protein Blot Stain (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions and visualized on an Eagle Eye (Stratagene, La Jolla, CA, USA). Developed films were scanned individually on a scanner (Scanjet 3770, Hewlett-Packard, Hamburg, Germany) with 1200 dpi and were saved as tiff files. Density of protein spots was measured using Scion Image (Scion Corporation, Frederick, MD, USA). Relative expression levels of proteins were calculated by normalizing to total protein, as determined by Sypro Ruby.

Antibodies

Antibodies used for Western blot and lysate microarrays are listed in Table 1.

Hierarchical clustering

Unsupervised hierarchical clustering was performed by using Cluster and Tree View software [28].

Self-organizing map analysis

The self-organizing map (SOM) analysis was performed using Viscovery Profiler 5 (Viscovery Software GmbH, Vienna, Austria, a subsidiary of Biomax Informatics AG, Martinsried, Germany). The SOM was calculated using the expression profiles of each protein of interest. All proteins were weighed equally. Automatic correlation compensation was used to calculate the map. Map segmentation was performed using a SOM-Ward clustering algorithm, generating three distinct groups. Group profiles were created including all proteins deviating from their mean expression level with a statistical confidence of above 80%.

Statistical analysis

The results were analysed by the Spearman rank correlation test, using SPSS version 13.0 (SPSS, Inc, Chicago, IL, USA). The significance level was set at $P > 0.05$.

Results

Cell culture analysis

To address the question whether EGFR activation effects Snail protein and/or mRNA expression, we examined the human endometrial carcinoma cell lines Ishikawa and Ishikawa ER-. Both cell lines have been established from an endometrial adenocarcinoma; the Ishikawa cells derive from the uterus, whereas the origin of the Ishikawa ER- cell line is not known. Both cell lines show an epithelial morphology and are EGFR-positive, but it is not known if the cell lines descend from the same patient.

We first analysed whether estrogen receptor signalling has an effect on Snail expression; however, stimulation with estrogen did not lead to changes in Snail protein levels (data not shown). EGF stimulation of serum-starved Ishikawa and Ishikawa ER- cells lead to up-regulation of Snail protein expression (Fig. 1). To identify the pathways mediating this effect, we inhibited EGFR, p38 MAPK (mitogen activated protein kinase), MEK/ERK (extracellular signal-regulated kinase) and PI3K (phosphatidylinositol 3-kinase) proteins (Fig. 2). In the Ishikawa cells (Fig. 2A), we found that the ERK pathway is involved in the EGF-dependent up-regulation of Snail

Table 1 Proteins analysed and antibodies used in this study

Protein	Antibody	Source
Phospho-AKT (Ser473)	#9271	New England Biolabs, Frankfurt am Main, Germany
AKT	C67E7, #4691	New England Biolabs
Cytokeratin 18	DC10, #4548	New England Biolabs
E-Cadherin	Clone 36 (AEC)	BD Biosciences, San Diego, CA, USA
Phospho-ERα (Ser118)	(16J4), #2511	New England Biolabs
ERα	578—595	Sigma Aldrich Chemie GmbH, Steinheim, Germany
Phospho-EGFR (Tyr1086)	ZMD.504	Invitrogen, Karlsruhe, Germany
EGFR	#2232	New England Biolabs
Phospho-GSK-3 ß (Ser9)	#9336	New England Biolabs
GSK-3 ß	27C10, #9315	New England Biolabs
Phospho-Her2 (Tyr1248)	#44—900	Invitrogen, Karlsruhe, Germany
Her2	Code A0485	DakoCytomation, Hamburg, Germany
Phospho-HSP 27 (Ser82)	#2401	New England Biolabs
HSP 27	G31, #2402	New England Biolabs
Phospho-NF-κB p65 (Ser536)	#3031	New England Biolabs
NF-κB p65	#3034	New England Biolabs
Phospho-p38 MAPK (Thr180/Tyr182)	12F8, #4631	New England Biolabs
p38 MAPK	#9212	New England Biolabs
Phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204)	#9101	New England Biolabs
p44/42 MAPK (ERK1/2)	#9102	New England Biolabs
Phospho-PAK1 (Thr212)	PK-18	Sigma Aldrich Chemie GmbH, Steinheim, Germany
PAK1	#2602	New England Biolabs
PTEN	#9552	New England Biolabs
Snail	9H2	Dr. Kremmer, München, Germany [44]

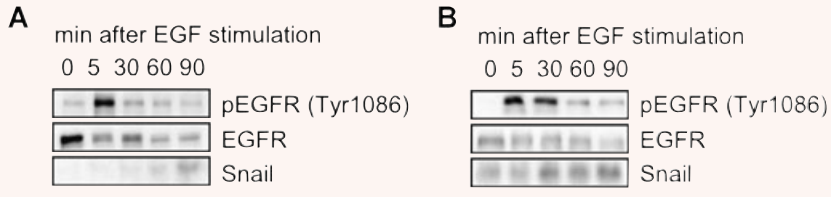

Fig. 1 Western blot analysis of Ishikawa (**A**) and Ishikawa ER- (**B**) cells. Cells were stimulated with EGF for 5–90 min. before protein extraction and 1D gel electrophoresis. Proteins analysed are indicated on the right.

protein expression; in the Ishikawa ER- cells (Fig. 2B), both AKT and ERK pathways participate in the up-regulation of Snail.

To examine if EGFR signalling also affects Snail mRNA expression levels, we performed quantitative real-time RT-PCR analysis of EGF-stimulated cells. We were surprised to find that Snail mRNA levels were only affected in Ishikawa cells; in Ishikawa ER- cells, EGF stimulation had no influence on Snail mRNA levels (Fig. 3A). Inhibition of EGFR and MEK/ERK pathways in Ishikawa cells led – as already seen for the protein expression – also to a reduction of Snail mRNA (Fig. 3B).

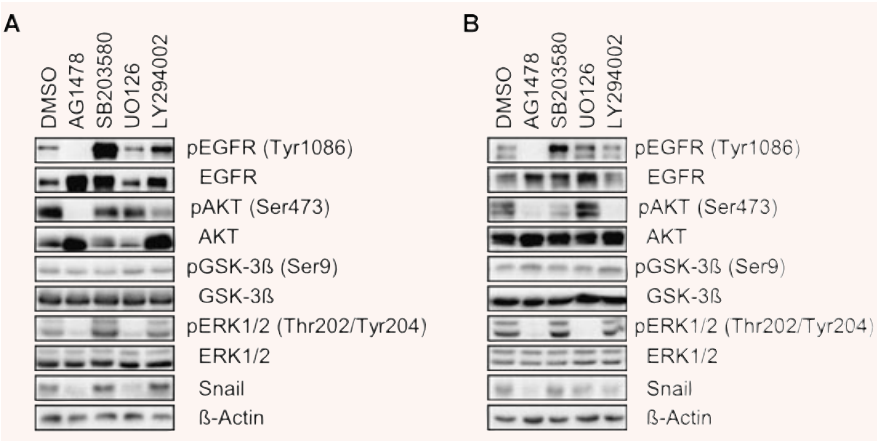

Fig. 2 Western blot analysis of Ishikawa (**A**) and Ishikawa ER- (**B**) cells. Cells were incubated with the specific protein kinase inhibitors AG1478 (inhibits EGFR), SB203580 (inhibits p38 MAPK), UO126 (inhibits MEK/ERK) and LY 294002 (inhibits PI3K) each dissolved in DMSO 60 min. prior to stimulation with EGF for 90 min.

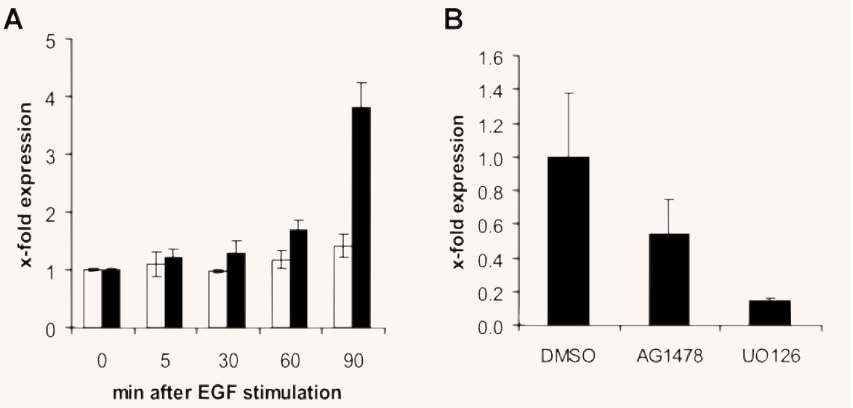

Fig. 3 Snail mRNA expression (real-time reverse transcription PCR) of Ishikawa (black bars) and Ishikawa ER- (white bars) cells. (**A**) Cells were stimulated with EGF for 5–90 min. before RNA extraction. Data are normalized to time-point zero (without stimulation). **(B)** Snail mRNA expression in Ishikawa cells after inhibiting the EGFR and ERK pathways. Cells were incubated 60 min. with AG1478 (inhibits EGFR) and UO126 (inhibits MEK/ERK) before stimulation with EGF for 90 min. Data are normalized to EGF-stimulated cells. The mean and S.D. of three independent experiments, each of which were performed in duplicate, are shown.

To broaden our mRNA study, we also applied Agilent microarray analysis with RNA extracted from Ishikawa cells 90 min. after EGF stimulation. Again, we found Snail mRNA among the genes that were up-regulated by at least two-fold compared to the unstimulated cells ($P = 0.018$, data not shown), confirming the real-time quantitative RT-PCR results. It was interesting that neither Slug, Twist, ZEB 1 nor Sip1 were found to be up-regulated in these cells after EGFR activation (data not shown). Because we did not see changes in Snail mRNA expression in the Ishikawa ER- cells after EGF stimulation using quantitative real-time RT-PCR (Fig. 3A), we applied Agilent microarray analysis only to Ishikawa cells.

Profiling of signalling proteins in primary endometrial cancers using protein lysate microarrays

To answer the question whether EGFR activation may be involved in Snail regulation in primary endometrial cancers, we applied protein lysate microarray technology. Before protein profiling, however, the specificities of all antibodies used in protein lysate microarray analysis were first confirmed. Therefore, we tested all 24 antibodies used in this study in Western blots with lysates from FFPE tissues using a recently established technology for extraction of full-length proteins from FFPE samples [29, 30]. All selected antibodies were found to be suitable for protein lysate microarray analysis (File S1).

After having demonstrated the specificities of all 24 antibodies, we performed protein lysate microarray analysis with protein extracts from 17 FFPE endometrial cancers in order to correlate Snail protein expression to abundances of a number of signalling proteins, including phosphorylated EGFR. Unsupervised hierarchical clustering analysis revealed that the samples were divided into two groups (Fig. 4). The first group shows Snail clustering close to the phosphorylated forms of EGFR and GSK-3β; the phosphorylated forms of p38 MAPK and AKT, HER2, PAK, ERK1/2, estrogen receptor α (ERα), the non-phosphorylated form of PTEN (phosphatase and tensin homolog deleted on chromosome ten) and Snail mRNA are also part of this cluster. The second cluster is composed of E-cadherin, Cytokeratin 18, the non-phosphorylated

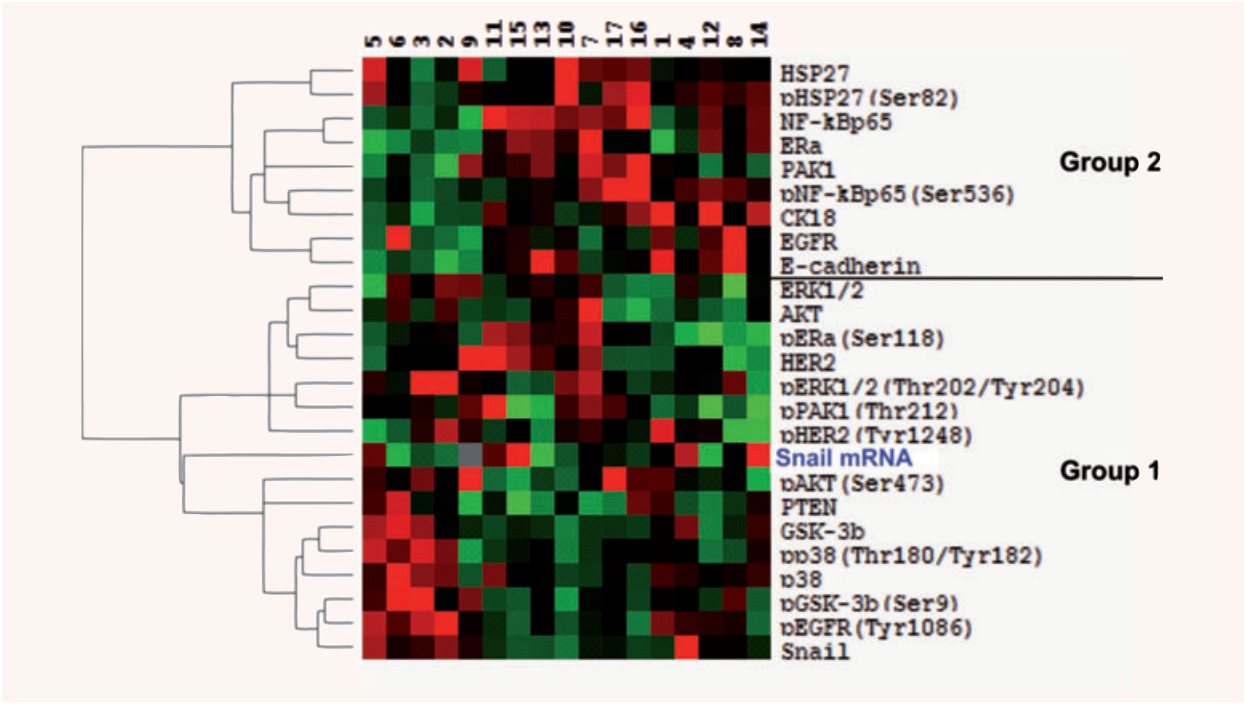

Fig. 4 Unsupervised hierarchical clustering image map. Twenty-four proteins (indicated in black) and Snail mRNA (indicated in blue) were used in the analysis of 17 separate tumours. Network groups (1-2) are indicated on the right.

form of EGFR, the phosphorylated forms of HSP 27 and NF-κBp65 and the non-phosphorylated forms of PAK1, estrogen receptor α, HSP 27 and NF-κBp65.

In primary endometrial cancers, we see a relation between Snail protein expression and the phosphorylated (Tyr1086) form of EGFR. In addition, the active, phosphorylated forms of GSK-3β, p38 and AKT may be involved in the regulation of Snail after activation of EGFR. We also see an inverse correlation between Snail and the two epithelial markers E-cadherin and Cytokeratin 18. The non-phosphorylated form of ERα showed an inverse correlation with Snail protein expression. This is in line with the fundings from Fujita *et al.* [31] but in contrast to Park *et al.* [32].

Cluster analysis does not provide a probability measure of the significance of the difference between clusters [33, 34]. This tool is, therefore, suitable for hypothesis generation, which is then assessed by other statistical methods [33]. For this reason, we applied the Spearman rank correlation test and found a statistical significant correlation ($P = 0.025$, $rho = 0.540$) between the phosphorylated form of EGFR and Snail expression.

In addition to cluster analysis and the Spearman rank correlation test, we performed additional multivariate statistical analysis based on SOM (File S2). The SOM was calculated solely based on the expression profile of each protein of interest. All proteins were weighted equally during map creation. The initial map was segmented using a SOM-Ward clustering algorithm. Three segments were identified (S1–S3). In all segments, p-EGFR showed segment-specific deviation from its mean expression in all experiments. For all segments, the statistical confidence of this deviation was above 95%. Segment S1 and S2 both showed under-representation of pEGFR; in contrast, S3 showed significant over-representation of pEGFR. It is notable that segment 3 showed – among other proteins – significant over-representation of Snail as well. In addition, E-cadherin and CK18 both showed significant under-representation in segment S3. Taken together, these data are in line with results obtained from unsupervised hierarchical clustering shown before.

Discussion

Our results revealed a correlation between EGFR signalling with increased abundances of the Snail protein, possibly regulated by the p38 MAPK, ERK1/2 and the AKT/GSK-3β pathways, and down-regulation of E-cadherin in primary endometrial tumours. In Ishikawa cells (Figs. 1–3, 5A), EGF stimulation leads to an activation of the ERK1/2 pathway and Snail mRNA and protein levels are increased. In contrast in Ishikawa ER- cells (Figs. 1–3, 5B), EGF leads to activation of the AKT and ERK1/2 pathways, which results in up-regulation of the Snail protein level but not of Snail mRNA level. Inhibition of EGFR prevents in both cell lines phosphorylation

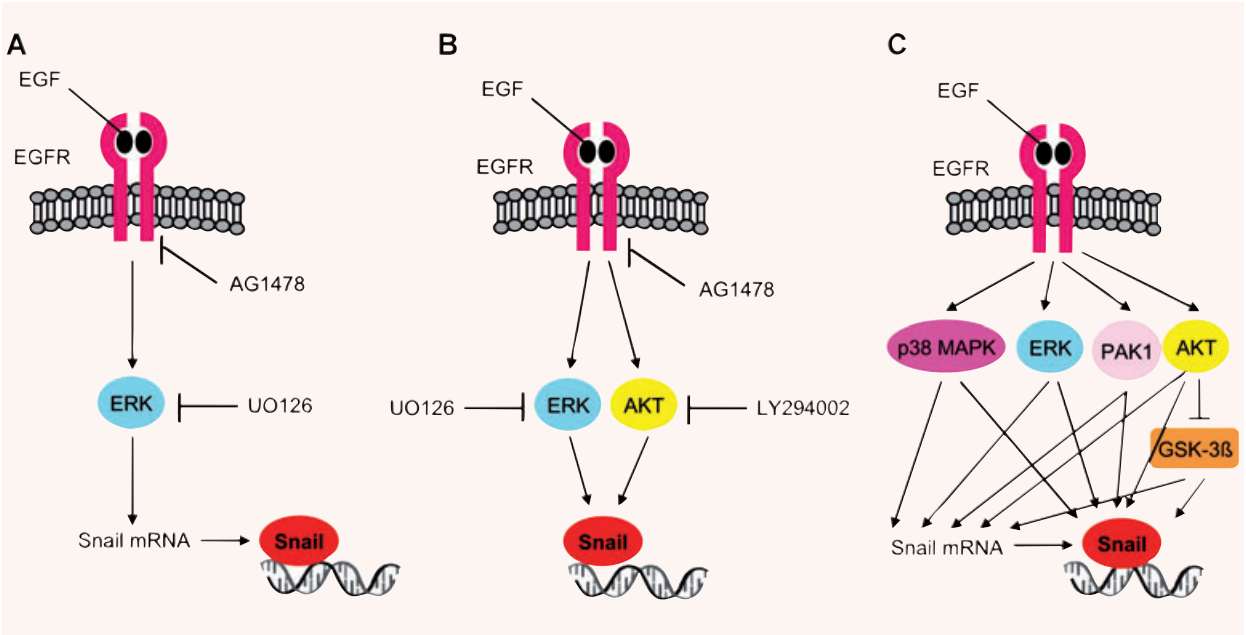

Fig. 5 Proposed model for EGFR-related signal transduction pathways regulating Snail in Ishikawa (**A**), Ishikawa ER- (**B**) and primary human endometrial tumours (**C**). EGF stimulates pathways leading to Snail's localization into the nucleus as well as pathways involved in reduced degradation of the protein. Snail shuttles into the nucleus after phosphorylation on Ser 246 by an unknown pathway, possibly with involvement of p38 MAPK, AKT, ERK1/2 and/or PAK1. After phosphorylation of GSK-3β on Serin 9 by AKT, GSK-3β is unable to phosphorylate and tag Snail for ubiquitination and proteasomal degradation. Our data clearly demonstrate that cell lines cannot fully reflect the activation status of signalling pathways in primary cancers. In the future, novel cancer treatments should target not only one but several signalling pathways.

of AKT, leading to the conclusion that AKT is a downstream target of EGFR. But only in Ishikawa ER- cells, inhibition of AKT results in Snail down-regulation. The increased phosphorylation of EGFR after inhibition of p38 MAPK leads to the suggestion that the cells try to compensate the inhibition of p38 MAPK by enhanced activation of EGFR. The elevated expression of EGFR may result from reduced internalization after ligand binding. In the primary endometrial tumours (Figs. 4, 5C), we see one large cluster (group 1) including Snail and the activated forms of EGFR, GSK-3β, p38MAPK, AKT, Her2, PAK1 and ERK1/2. Each of the tumours shows a different protein expression profile indicating a complex, patient-specific regulation of Snail in human endometrial cancers.

Zohn *et al.* reported that p38 activation *in vivo* is required for down-regulation of E-cadherin protein during gastrulation in mice. p38 regulates E-cadherin expression independently of the transcriptional repressor Snail [35]. In our protein lysate study, we also see an inverse correlation between p38 and E-cadherin expression. In human dermal fibroblasts, EGF treatment resulted in activation of p38 MAPK [21]. In contrast to these findings, we did not see an influence of the p38 MAPK pathway on Snail mRNA and protein levels in Ishikawa and Ishikawa ER- cells. In our analysis with primary endometrial carcinoma tissues, expression of Snail clustered very close to the phosphorylated forms of EGFR, p38, AKT and GSK-3β, indicating that these pathways may play an important role in this disease. In addition, we found an inverse clustering between Snail and HSP27, which is in line with results reported recently [36]. However, in the Ishikawa and Ishikawa ER- cell lines, GSK-3β phosphorylations were not influenced by EGF. Lu *et al.* already reported no significant change in the level of phosphorylated GSK-3β in response to EGF in A431 cells [23].

In this study, we also could confirm that the ERK pathway is involved in the activation of Snail in both Ishikawa cell lines and in primary endometrial tumours, as reported by others using promoter analysis [37]. It was interesting that we saw in cluster analysis of the endometrial primary tumours a close association of the active forms of PAK1 and ERK1/2. Sundberg-Smith *et al.* reported that adhesion signalling induces a direct association between ERK and PAK1. They showed that ERK2 phosphorylates PAK1 on Thr212 *in vitro* in rat smooth muscle cells, which may provide negative feedback inhibition of ERK signalling [38]. PAK1 was also reported to phosphorylate Snail and to promote its localization into the nucleus in human breast cancer cell lines [16]. In our study, however, we did not find any EGF-related correlation between PAK1 and Snail in the cell lines but found the active form of PAK1 clustering with Snail in primary tumours. We could not

find a positive correlation between NF-κB p65 and Snail expression as it was reported before in LX-B cells [39]. But as already described by Saegusa *et al.*, we also found a positive correlation between NF-κBp65 and E-cadherin and an inverse correlation between NF-κB and Snail [40]. It should be kept in mind, however, that a single cell line may not be representative for a complex disease like cancer which involves many different subtypes and a close interaction between tumour and stromal cells (Fig. 5). In our study, we also found different expression profiles in each of the 17 tumours examined (Fig. 4), emphasizing the need for individualized therapy regimens targeting several signalling pathways at the same time.

A general observation made in this study was that apparent expression of phosphorylated and non-phosphorylated forms of a protein do not necessarily correlate (Fig. 4). Different affinities of the antibodies for each form may explain at least in part this observation. In line with this observation is the fact that we could detect pEGFR (Tyr1086) in the breast cancer cell line MDA-MB-435S after stimulation with EGF, but we could not prove EGFR expression in these cell line (Hipp S, unpublished observation).

Previous studies reported that decreased E-cadherin expression correlated with loss of differentiation and depth of myometrial invasion in human endometrial carcinoma [5]. Blechschmidt *et al.* already found that abnormal E-cadherin expression correlated with positive Snail immunoreactivity in metastases of endometrial carcinomas and suggested that Snail might play a role in tumour dedifferentiation and progression [18]. With the protein microarray data reported in our study here, we confirm the inverse correlation between Snail and E-cadherin expression in endometrial cancers and found also Cytokeratin 18 correlating with E-cadherin. We did not find a statistical significant correlation between Snail and tumour grade, UICC and the age of the patients.

Her2/neu overexpression occurs in progression and dedifferentiation in about 15–20% of endometrial cancers and often correlates with a higher tumour grade and a poor survival [41, 42]. Accumulating evidence suggests that activated Her2 leads to the onset of intracellular signalling events involving ERK [43]. We also found a correlation between the phosphorylated forms of Her2, PAK1 and ERK1/2, suggesting that this pathway may also be involved in the pathogenesis of human endometrioid endometrial carcinoma (Fig. 4).

In conclusion, in this pilot study we found a statistical significant correlation between activation of EGFR and Snail protein overexpression in primary endometrial cancer tissues, most likely regulated by the p38 MAPK, ERK1/2 and the AKT/GSK-3β pathways. Our data have now to be confirmed in a larger series and in other cancers as well. In addition, other epithelial-mesenchymal-transition regulators (*e.g.* Slug, E12, ZEB2) need to be integrated in these studies. In the near future, when more antibodies against different E-cadherin repressors are available, the interplay of these EMT regulators and the exact signal pathways involved in their activation can be analysed in a variety of primary human cancers using our approach which integrates quantitative protein analysis in formalin-fixed clinical tissues.

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Conflict of interest

KFB is a named inventor of a patent related to protein extraction from formalin-fixed and paraffin-embedded tissues.

Supporting information

The following supporting information is available for this article:

File S1. All but one of the 24 antibodies used in this study were tested in Western blot with lysates from endometrioid endometrial cancers. Her2 immunoreactivity was evaluated using breast cancer tissues. (A) Most antibodies tested showed one single band at the expected size. (B) For testing the specificity of antibodies with more than one band in the Western blot, lysates from one high and one low expressing tumour were analysed to show that all bands detected correlate with the expression level of the protein of interest.

File S2. (A) Visualization of parameter values (protein expression) for calculated self-organizing map (SOM). Map segmentation based on SOM-Ward clustering is indicated by bold segment borders. (B) Group profiles for segment S3. The bar graph represents the segment-specific deviation from the mean over all experiments, measured in standard deviations. Only proteins deviating with a statistical confidence of 80% or higher are shown. The insert illustrates the three segments. (C) Group profiles for segments S1–S3. The bar graph represents the segment-specific deviation from the mean over all experiments, measured in standard deviations. Only proteins deviating with a statistical confidence of 80% or higher are shown.

Table S1. Summary of tumour grading, staging and age of the patients

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(This link will take you to the article abstract).

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