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Gefitinib-responsive EGFR-positive colorectal cancers have different proteome profiles from non-responsive cell lines

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Abstract

Biomarkers that predict response to therapy with inhibitors of epidermal growth factor receptor (EGFR) tyrosine kinase remain largely uncharacterized. In order to define proteins involved in potential resistance mechanisms, we examined the effect of gefitinib (ZD1839, Iressa) in the EGFR-positive colon cancer cell lines Caco-2, DiFi, HRT-18 and HT-29. None of them exhibited an activating mutation in exons 19 or 21 of EGFR. Proteome profiling with two-dimensional polyacrylamide gel electrophoresis followed by mass spectrometry revealed 12 proteins differentially expressed in responsive and non-responsive cells. These proteins are involved in metabolic pathways, partially relevant in malignant growth and four of them are known to interact with the EGFR signalling pathway. Ubiquitin carboxyl-terminated hydrolase isozyme L1 (UCH-L1) and galectin-3 are overexpressed in the responsive cell line Caco-2, whereas fatty acid-binding protein (E-FABP) and heat shock protein (hsp) 27 are expressed more in the resistant cell lines HRT-18 and HT-29 suggesting a role in non-responsiveness of cells to gefitinib.

Keywords: Colorectal cancer; Gefitinib; Resistance; Proteomics; EGFR

1. Introduction

Colorectal cancer (CRC) is a leading cause of cancer death in both men and women throughout the world. Although chemotherapy has improved the outcome of patients with metastatic disease during recent years, new therapies with novel mechanisms of action are warranted. The development of various therapeutic strategies directed against specific target molecules expressed by the malignant cells is a promising strategy [1]. Approximately 70% of human colon carcinomas have been shown to express the epidermal growth factor receptor (EGFR), and is correlated to more aggressive disease and poorer prognosis [2].

EGFR (ErbB-1 or HER-1) is a member of the ErbB family of receptor tyrosine kinases (RTKs) and consists of an extracellular ligand-binding domain, a single transmembrane region, and an intracellular domain with tyrosine kinase activity [3]. Activation of the receptor occurs after binding of its ligands, such as transforming growth factor-α (TGF-α) or EGF, resulting in receptor dimerisation with either another EGFR molecule or another ErbB receptor. Dimerisation triggers activation of the intracellular kinase domain, autophosphorylation of tyrosine residues in the intracellular domain, and subsequent recruitment and activation of

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downstream signalling molecules. Aberrant EGFR activity has been associated with tumour growth in several aspects, including the promotion of cell proliferation, angiogenesis, invasion and metastasis, and survival. Two critical signalling routes activated by EGFR involve the Ras–Raf–MEK–ERK and the PI3K–PDK1–Akt kinase pathways, which are implicated in cell proliferation, survival, and gene expression. Strategies aimed at interrupting this signalling pathway include monoclonal antibodies directed against the ectodomain of the receptor or small-molecule tyrosine kinase inhibitors (TKIs).

Gefitinib (ZD1839, Iressa®) is an orally active, selective, and reversible EGFR-TKI that chemically belongs to the class of anilinoquinazolines [4]. *In vitro* effects of gefitinib as a single agent are mainly cytostatic, although cytotoxic effects have also been observed in a few cases. Gefitinib has been approved for the treatment of pretreated advanced lung cancer in a number of countries around the world.

In colorectal cancer cells, gefitinib has been shown to exert both *in vitro* and *in vivo* antitumour activity as monotherapy or in combination with cytotoxic agents such as irinotecan and oxaliplatin [5–9].

The impact of EGFR expression levels on drug sensitivity to EGFR blockers is still an issue since preclinical studies and clinical data have shown no correlation with response [10,11]. Several molecular pathways have been investigated that may be involved in the sensitivity of cells to gefitinib, including phosphorylation of EGFR and downstream receptor-dependent molecules such as mitogen-activated protein kinase (MAPK), Akt, or p27 [12,13], but their significance as a biomarker predicting sensitivity towards EGFR-blocking agents has not yet been proven.

The major challenge is posed by the identification of subsets of patients who will benefit from therapy with EGFR-blocking agents. Recently it was shown that a subgroup of patients with non-small cell lung cancer demonstrates specific mutations in the EGFR gene, which correlate with clinical responsiveness to gefitinib [14]. These mutations cause increased growth factor signalling and may predict sensitivity to signal transduction inhibitors, but have not yet been described for CRC.

To define tumour proteins that may predict sensitivity to gefitinib we selected four colorectal cancer cell lines with a high expression of EGFR but a differing response pattern to gefitinib. Comparison of proteome profiles of Caco-2, DiFi, HRT-18 and HT-29 cell lines after 2-D PAGE revealed twelve proteins that were differently expressed in responsive and non-responsive cell lines. Our data suggest that proteome-based technologies may be a new tool for understanding the complex network of EGFR inhibition and interactions in malignant cells after treatment with anticancer agents such as EGFR-TKIs.

2. Materials and methods

2.1. Cell cultures and growth inhibition assay

The colorectal cancer cell lines Caco-2, HRT-18 and HT-29 were purchased from the American Type Culture Collection. Caco-2 and HT-29 cells were grown in Minimum Essential Medium (Eagle) (PAA Laboratories GmbH, Linz, Austria) supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 50 U/mL penicillin, 50 μg/mL streptomycin, and 10% fetal calf serum (v/v) (Sigma-Aldrich, Vienna, Austria). HRT-18 cells were maintained in RPMI 1640 medium (PAA Laboratories GmbH, Linz, Austria) containing 2 mM L-glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin, and 10% fetal calf serum (v/v). DiFi human colon adenocarcinoma cells were maintained in DMEM-F12 medium (Invitrogen GmbH, Lofer, Austria) supplemented with 2 mM L-glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin, and 10% fetal calf serum (v/v) [15]. Cultures were incubated in a 5% CO_2 humidified atmosphere.

On day 1, from each cell line 5000 cells per well were plated in 96-well plates. On day 2, various concentrations of gefitinib (0.05, 0.1, 0.5 and 1 μM) were added with four different exposure times each (24, 48, 72 and 96 h). These concentrations reflect the minimum concentration that induces growth arrest in sensitive colorectal cancer cells and the mean plasma concentration (0.4–1.4 μM) received in patients after daily, oral administration of 250–600 mg gefitinib [14].

Growth and viability of all four gefitinib-treated cell lines was compared with control cells by means of sulforhodamine B anionic dye staining. Cultured cells were fixed with 50 μL/well ice-cold 50% trichloroacetic acid (v/v) at 4 °C overnight after 24, 48, 72 and 96 h of treatment. After washing five times with water and air-drying for approximately 20 min, cells were stained with 100 μL of 0.4% sulforhodamine B (w/v) (Sigma-Aldrich, Vienna, Austria) in 1% acetic acid (v/v) for 15 min. Subsequently, plates were washed five times with 1% acetic acid (v/v), air-dried and the dye resuspended in 100 μL of 10 mM Tris buffer (pH 10.5). Dye was quantified with a microplate reader (SPECTRAFluor Plus, Tecan, Austria) at 510 nm. Cell proliferation was determined as percent of control.

For statistical evaluation, mean values and standard deviation were calculated from three independent experiments; significance was determined with the paired Student *t*-test.

2.2. Analysis of EGFR expression and EGFR mutations

For analysis of EGFR expression by flow cytometry (FACSCalibur; Becton Dickinson, San Jose, USA) 1×10^6 cells per cell line per tube were pelleted at 200×*g*, washed twice with PBS and stained with 4 μg/mL mouse

monoclonal IgG_{2a} anti-EGFR (528) PE: sc-107 PE antibody (Santa Cruz Biotechnology, Heidelberg, Germany). An equivalent amount of PE-conjugated IgG_{2a} mAb (DakoCytomation GmbH, Vienna, Austria) was used as isotype control. Propidium iodide (Sigma Chemical Co., St. Louis, MO, USA) was added to all assays to exclude dead cells. From each sample, ten thousand life events were collected by FACSCalibur.

To detect potential mutations in exons 19 and 21 of the EGFR gene in Caco-2, DiFi, HRT-18 and HT-29 cells, DNA was extracted from cell lines using the BioRobot M48 workstation with MagAttract technology as described elsewhere (Qiagen, Germany). Polymerase chain reaction and sequencing was performed using primer pairs and conditions described by Lynch and colleagues [14].

2.3. Western blot analysis

EGFR phosphorylation status was determined only in Caco-2 and HRT-18 cells, because in HT-29 and DiFi cells activation of EGFR has already been published [16–18]. For Western blotting, Caco-2 and HRT-18 cells were lysed for 15 min at 4 °C in RIPA lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/mL leupeptin, 5 μg/mL aprotinin, 1% NP40 (w/v), 0.5% deoxycholic acid sodium salt (w/v), 0.1% sodium dodecyl sulfate (SDS) (w/v)) and sonicated. After centrifugation at 12000×*g* for 10 min at 4 °C, supernatant was collected and protein concentrations were determined with a commercial protein assay (BioRad Laboratories, Hercules, CA, USA); 40 μg of protein per lane was separated by 12% SDS polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). Protein loading was controlled by Ponceau red staining of membranes. After blocking for one hour in Tris-buffered saline (TBS) supplemented with 5% non-fat milk (v/v) and 0.1% Tween 20 (w/v) (Sigma-Aldrich Vienna, Austria), membranes were incubated for one hour at room temperature with antibody against the activated form of human EGF receptor (BD Biosciences, San Diego, USA) or with α-tubulin (Oncogene Research, Cambridge, USA) as loading control. Membranes were washed three times in TBS-Tween and incubated for one hour with horseradish peroxidase-conjugated anti-mouse secondary antibody (Amersham, Les Ulis, France). Immunodetection was performed with a chemiluminescence system (ECL, Amersham Biosciences, Vienna, Austria).

2.4. Two-dimensional polyacrylamide gel electrophoresis

For sample preparation Caco-2, DiFi, HRT-18 and HT-29 cells were scraped and harvested by centrifugation at 4 °C for 10 min at 200×*g*. After washing twice with PBS, cells were lysed for 15 min at 4 °C in lysis buffer (10 mM Tris–HCl, pH 7.5, 25 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/mL leupeptin, 5 μg/mL aprotinin, 0.1% NP40 (w/v)) and sonicated. To remove the nuclear fraction, the homogenate was centrifuged at 3000×*g* for 10 min at 4 °C, and the supernatant collected in a new tube. In order to avoid contamination with salts or nucleic acids, lysates were processed using trichloroacetic acid at a final concentration of 10% (v/v) (Serva, Heidelberg, Germany). Precipitated proteins were washed three times with cold acetone and resuspended with isoelectric lysis solution (7 M urea, 2 M thiourea, 4% CHAPS (w/v), 0.5% IPG buffer (v/v)). Protein concentrations were determined with a commercial protein assay (BioRad Laboratories, Hercules, USA).

For first-dimension isoelectric focusing, 60 μg of protein per sample was diluted to 450 μL with rehydration buffer and loaded on immobilized pH gradient strips. Active rehydration (50 V) was performed at 20 °C for 12 h. For pH 3–10NL IPG strips, isoelectric focusing was performed at 250 V for 30 min, 500 V for one hour, 2000 V for one hour and finally at 8000 V/h until 64000 V was reached in total. pH 6–9 IPG strips were processed at 250 V for 30 min, 500 V for one hour, 2000 V for one hour and at 8000 V/h until 110000 V.

For second-dimension, samples were separated on 12.5% polyacrylamide gels with the Ettan Dalttwelve System following the standard procedure recommended by the manufacturer (Amersham Biosciences, Vienna, Austria). After electrophoresis, gels were silver-stained and scanned using an ImageScanner (Amersham Pharmacia Biotech AB Uppsala, Sweden). Data analysis was performed with IMAGEMASTER 2-D ELITE V 4.01 software (Amersham Pharmacia Biotech AB Uppsala, Sweden). To obtain a standard gel for each individual cell line, background subtraction, spot detection and matching were performed using gels from three runs. These standard gels were then matched to yield information about differentially expressed proteins.

2.5. Identification of proteins by mass spectrometry

Protein analysis was performed as previously published [19]. Protein digests were separated using capillary HPLC connected on-line to an LCQ ion trap instrument (ThermoFinnigan, San Jose, USA) equipped with a nanospray interface. MS/MS spectra were searched against a human database using SEQUEST (LCQ BioWorks; ThermoFinnigan).

3. Results

3.1. Growth inhibitory effect of gefitinib

Treatment of the colorectal cancer cell lines Caco-2, HRT-18, DiFi and HT29 with various concentrations

of gefitinib (0.05, 0.1, 0.5 and 1 μM) for 24, 48, 72 and 96 h revealed sensitivity to this agent in Caco-2 and DiFi, whereas growth of HRT-18 and HT-29 was not inhibited (Fig. 1). At concentrations of 0.5 and 1 μM, Caco-2 cells showed a time-dependent growth inhibition of 104.2 ± 16.3 and 113.9 ± 12.7%, respectively, of cells surviving after 24 h, 79.5 ± 12.5 and 80.1 ± 9.3% after 48 h, 59.7 ± 5.8 and 61 ± 6.1% after 72 h, and 45.05 ± 9.1 and 40 ± 12.3% after 96 h of incubation. In DiFi cells, growth was already reduced after exposure to 0.05 and 0.1 μM gefitinib (Fig. 1). In comparison with Caco-2, DiFi cells displayed similar time-dependent growth inhibition at concentrations of 0.5 and 1 μM, with 69.1 ± 6.9 and 68.3 ± 7,4%, respectively, of cells surviving after 24 h, 60.3 ± 2.7 and 57.7 ± 3.8% after 48 h, 51.6 ± 2.8 and 46.6 ± 3.2% after 72 h, and 42.0 ± 8 and 37.9 ± 4.7% after 96 h of incubation.

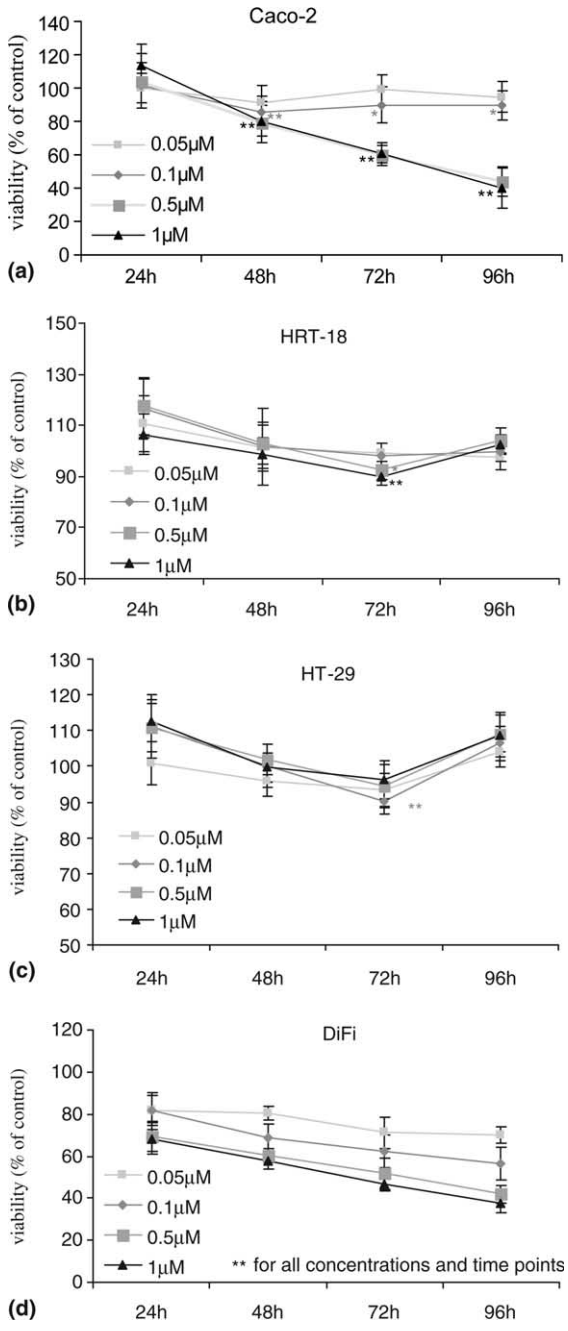

Fig. 1. Effect of gefitinib on the cell growth of EGFR-positive cell lines: (a) Caco-2; (b) HRT-18; (c) HT-29; (d) DiFi. Cells were treated with various concentrations of gefitinib for 0–96 h. Only growth of Caco-2 and DiFi cells was inhibited in a time-dependent manner. Data are given as mean and standard deviation obtained from three independent experiments with * = $P < 0.01$ and ** = $P < 0.001$ *versus* untreated cells.

3.2. EGFR expression and EGFR mutations

The cancer cell lines revealed high expression of EGFR, with 77.1% of Caco-2 cells, 97.8% of HRT-18 cells and 78.1% of HT-29 cells being EGFR-positive. A similar EGFR expression on DiFi cells has already been shown before [18]. Mean fluorescence intensity for EGFR staining was 19.5 for untreated Caco-2 cells, 32.2 for HRT-18 cells and 66.6 for HT-29 cells.

No activating EGFR mutations were found in exon 19 or 21 of Caco-2, DiFi, HRT18 or HT-29 cell lines. A polymorphism in intron 19 (IVS19 + 96A > G) was detected homozygously in Caco-2.

3.3. Functional EGFR activation

To determine the functional activity of EGF receptors in Caco-2 and HRT-18 cell lines, these cells were stimulated with EGF (10 nM) and phosphorylation status of EGFR was evaluated by Western blotting. Results of immunoblotting with an antibody specifically directed against the phosphorylated EGFR on Caco-2 and HRT-18 cells are shown in Fig. 2. Maximum phosphorylation of EGFR in Caco-2 cells was achieved after

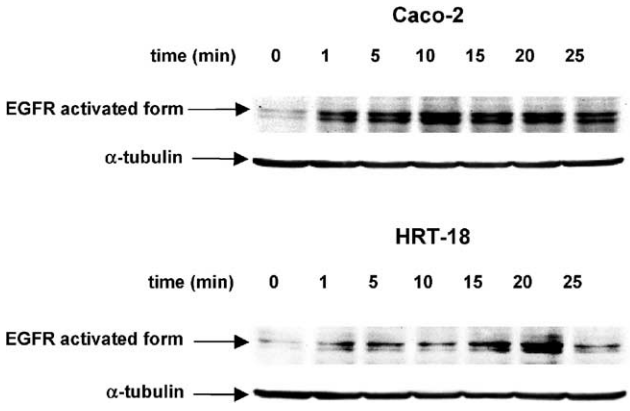

Fig. 2. Functional EGFR activation in Caco-2 and HRT-18 cells. Cells were serum-starved overnight, followed by 10 nM EGF stimulation. Samples for Western blot analysis were collected before treatment and at 1, 5, 10, 15, 20 and 25 min after EGF stimulation. Maximum phosphorylation of EGFR was achieved in Caco-2 cells after 10 min and in HRT-18 cells after 20 min.

Table 1
Characteristics of the proteins identified by 2-D PAGE

Target protein (accession no./MW kDA)	Sequence coverage (%)	Function	Caco-2/DiFi/HRT-18/HT-29 expression
Ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCH-L1) (P09936/24.8)	70.5	Involved in deubiquitination, association with p27, prognostic factor in some cancers**	Expressed only in Caco-2
Glutathione-S-transferase P (GSTP) (P09211/23.4)	66.3	Catalyzes glutathione conjugation, protection from cytotoxic and radiation damage, regulation of stress kinases, antiapoptotic effects**	6/5/1/3
Nicotinate-nucleotide pyrophosphorylase (QPRTase)(Q15274/30.8)	35.6	Essential for de novo synthesis of NAD (quinolinate catabolism)	12/4/2/1
Microtubule-associated protein RP/EB family member 1 (Q15691/30.0)	59.3	Regulates microtubule dynamics and chromosome segregation, direct interaction with APC tumor suppressor protein**	2.9/1/1/1
Galectin-3 (P17931/26.2)	34.2	Implicated in cell growth, differentiation, adhesion and apoptosis**	4.1/1/1/1
Protein kinase C inhibitor protein-1 (PKCI-1) (P31946/27.9)	46.6	Originally thought to inhibit protein kinase C, PKCI deficiency enhances tumorigensis**	3.7/3/1.4/1
Epidermal fatty acid-binding protein (E-FABP) Q01469/15.2	76.4	Transport of fatty acids and lipid ligands, modulation of mitosis, cell growth, differentiation, protein kinase C activation**	0/1/2.6/4.3
Heat shock 27 kDa protein (HSP 27) (P04792/22.8)	44.1	Inhibits actin polymerization, associates with tubulin, antiapoptotic activity**	1/0/3.5/5
Profilin I (P07737/15.1)	32.4	G-actin sequestration and inhibition of actin polymerization**	1/0/3.9/3.8
Inorganic pyrophosphatase(Ppase) (Q15181/32.7)	62.4	Important role in energy metabolism, catalyzes hydrolysis of inorganic pyrophosphate to ortho-phosphate	1/1.9/3.7/3.3
Phosphoserine aminotransferase (PSAT) (Q9Y617/40.4)	46.8	Required for synthesis of nucleotide precursors (linked to replication), catalyzes glycolate to phosphoglycerate	1/1.2/20.9/15
Proteasome subunit alpha type 7 (PSA 7) (O14818/27.9)	43.5	Belongs to 20S proteasome, may alter activity of proteasome complex (*e.g.*, regulation of transcriptional factors in cancer cells)**	1/0/3/3

Protein identities, accession number (no), sequence coverage, molecular weight (MW), function and relative expression of identified proteins. Differences in protein expression are indicated as ratio between means of protein expression in Caco-2, DiFi, HRT-18 and HT-29 cell lines. Known involvement in cancer is expressed as **.

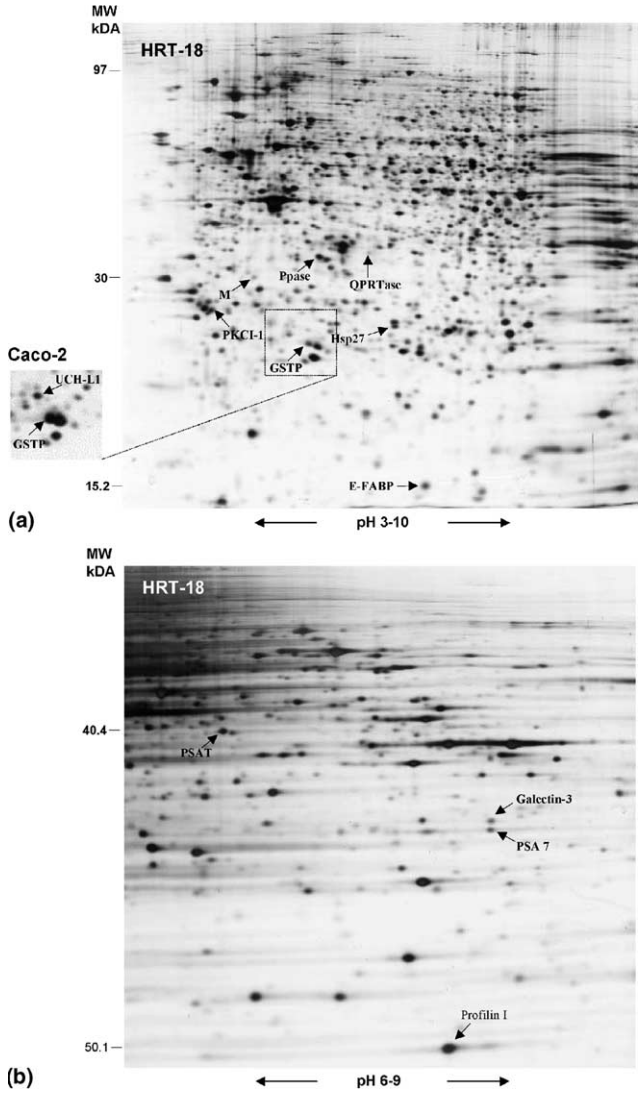

Fig. 3. Localisation of identified target proteins on representative silver-stained 2-D PAGE gels: (a) eight proteins were found within pH range 3–10. UCH-L1 was exclusively present in the Caco-2 cell line, which is illustrated in the corresponding excerpt; (b) four target proteins were located within pH 6–9. Accession number, molecular weight and differential expression of all proteins are listed in Table 1. M, microtubule-associated protein RP/EB family member 1.

10 min and in HRT-18 cells after 20 min. Induction of EGFR phosphorylation in HT-29 and DiFi has been shown previously [16–18].

3.4. Identification of proteins using 2-D PAGE

In order to identify proteins with various levels of expression in the gefitinib-responsive cell lines Caco-2 and DiFi *versus* the resistant cell lines HT-29 and HRT-18, we examined the protein profiles of whole cell extracts after removal of the nuclear fraction by 2-D PAGE. Silver-stained gels were analysed with IMAGEMASTER 2-D ELITE V 4.01 software for intensity of protein spots. Mean values of relative spot volumes, standard deviation and differences in expression were calculated from at least three independent experiments. When the expression level of individual proteins between responsive and non-responsive cells differed by a ratio of more than 2.0, spots were quantified according to their relative volume. Among the approximately 1900 protein spots detected per cell line, twelve proteins displayed quantitative differences in expression between the sensitive and the resistant cell lines and also had a sufficient level of expression to allow further processing. All these proteins were analysed using mass spectrometry in conjunction with the SWISS-2-D PAGE and SIENA-2-D PAGE protein databases to assign putative identities.

Identified proteins as well as their physical parameters and biologic functions are summarised in Table 1 and Fig. 3. They belong to various structural or functional families such as proteins with detoxification function, metabolic enzymes, cytoskeleton-related proteins, cell cycle regulators, chaperones, proteins with calcium channel activity as well as with unknown function. The expression level of none of these proteins was changed after treatment with gefitinib.

4. Discussion

The epidermal growth factor receptor (EGFR) is expressed in the majority of advanced colorectal cancers (CRC), and higher levels of EGFR are inversely correlated to survival in these patients [20]. Therefore, targeting of EGFR with tyrosine kinase (TK) inhibitors is a promising therapeutic approach in these tumours.

Clinical trials performed with the EGFR-TKI gefitinib in patients with EGFR-positive metastatic colorectal carcinoma showed inhibition of EGFR signalling and reduced cell proliferation in some but not all tumour biopsies [9]. This study demonstrates no clear correlation between the level of EGFR expression and changes in proliferation index or apoptosis. Based on clinical data with gefitinib therapy in patients with solid tumours like NSCLC, head and neck cancer, colorectal cancer, prostate and breast cancer [21], remissions occur only in a subgroup of patients and are of limited duration, suggesting the existence of primary and secondary resistance mechanisms. Recently it was shown that constitutively active Erk and Akt kinase pathways may protect cells from apoptosis induced by EGFR-targeting agents [22]. None of these parameters has yet been validated as a biomarker to predict positive treatment outcome. Therefore, the molecular mechanisms involved in gefitinib-mediated anticancer effects remain poorly understood. In a subgroup of patients with non-small cell lung cancer responsiveness to TKIs correlated with specific mutations in the kinase domain of EGFR

[14]. These mutations were not found in CRC [23] and were not present in Caco-2, DiFi, HRT-18 or HT-29 cell lines, all exhibiting a high expression of functional EGFR. The single nucleotide polymorphism found in Caco-2 is of unknown relevance and has been found with an allele frequency of 0.2 in 70 head and neck squamous cell carcinoma (data not published).

In order to define proteins that might be involved in response or resistance to therapy with gefitinib, we investigated these four colorectal cancer cell lines using a proteomic approach. While growth of Caco-2 and DiFi cells was inhibited in a time-dependent fashion, proliferation of HRT-18 and HT-29 cells was not influenced.

We were able to define a spectrum of markers that are differentially expressed in the gefitinib-sensitive cell lines Caco-2 and DiFi as compared to the gefitinib-resistant cell lines HRT-18 and HT-29. Analysis of the proteome profile by 2-D PAGE revealed twelve proteins with significant difference in expression level. All these proteins are involved in metabolic pathways, and nine of them have previously been shown to be involved in malignant growth.

Ubiquitin carboxyl-terminated hydrolase isozyme L1 (UCH-L1) is exclusively expressed in Caco-2 cells. This enzyme is involved in the processing of ubiquitinated proteins and is a marker for invasive colorectal carcinoma, pancreatic and lung cancer [24–26]. Liu and colleagues [27] demonstrated that UCH-L1 enzymatic activity is antiproliferative. UCH-L1 interacts with p27, a cyclin-dependent kinase inhibitor, which induces cell cycle arrest in the G1 phase [28]. In human myeloma cells upregulation of UCH-L1 was associated with cell cycle retardation and overexpression of p27 [29]. There is strong evidence from *in vitro* studies that up-regulation of p27 is essential for growth inhibition by EGFR-TKIs [30]. Acting further upstream in the EGFR pathway, galectin-3 was shown to dephosphorylate Akt in a cell culture model [31]. This molecule is also overexpressed in the gefitinib-responsive cell line Caco-2. Therefore, both UCH-L1 and galectin-3 may be involved in the gefitinib-mediated cytostatic effects in Caco-2 cells only.

Furthermore, glutathione S-transferase P (GSTP) showed greater expression in Caco-2 and DiFi cells. This enzyme belongs to the glutathione S-transferase superfamily and is responsible for detoxification of chemicals and acquired drug resistance [32]. Higher expression of GSTP in Caco-2 and DiFi cells did not cause resistance to gefitinib. GSTP is also involved in the development of colorectal carcinoma [33]. Recently, antiapoptotic effects of GSTP caused by crosstalk with p38, Erk, IKK and JNK have been demonstrated [34]. Despite these possible interactions with the EGFR pathway, our model did not show overexpression of GSTP to be associated with therapy resistance.

Whether further proteins with a higher expression level in Caco-2 and DiFi (Table 1) interfere with the antiproliferative effect of gefitinib is unclear, because knowledge of their potential role in malignant growth is limited.

In principle, all proteins expressed at a higher level in cells non-sensitive to the TK inhibitor might display candidates involved in resistance mechanisms. The fatty acid-binding protein E-FABP is overexpressed in HRT-18 and HT-29 cells. This protein is involved in modulation of mitosis, cell growth and differentiation [35]. Fatty acids are transported by FABP and hydroxylated. Hydroxylated fatty acids modulate the EGF signalling pathways and stimulate both the EGF receptor and protein kinase C (PKC) [36]. As PKC is a mediator of the PI3K/Akt pathway that prevents cell apoptosis, increased expression of E-FABP may be involved in mechanisms contributing to the non-responsiveness of HRT-18 and HT-29 to gefitinib treatment. Overexpression of E-FABP has also been associated with multidrug resistance in human adenocarcinoma of the pancreas [37]. In this context it is interesting to note that the PKC inhibitor PKCI-1 was found to be less expressed in HRT-18 and HT-29 than in Caco-2 and DiFi cells.

Up-regulation of PKC activity causes phosphorylation of Hsp27 [38], an antiapoptotic protein expressed at a higher level in the two resistant cell lines. Hsp27 is a cytoplasmatic chaperone participating in stress resistance, cell growth, differentiation and microfilament organization [39]. Overexpression of hsp-27 is associated with aggressive behaviour of various tumours and correlates with resistance to chemotherapeutic agents [40,41]. Hsp27 confers protection against cell death through complex mechanisms, including a direct interaction with cytochrome C and with two effectors of the EGFR pathway, namely Akt and p38 MAPK [42]. Crosstalk of chaperones with ErbB receptor downstream signalling has recently been demonstrated for Hsp90 [43]. Its inhibition down-regulates Akt-kinase and induces HER2 degradation. Our data suggest that Hsp27 might also play a role in inhibiting antiproliferative effects of gefitinib and could be a target for combination therapy. Involvement of Hsp27 in resistance mechanisms has recently been shown in lymphoma cells, where Hsp27 antisense strategies could reverse resistance to the proteasome inhibitor bortezomib [44].

In summary, two-dimensional gel electrophoresis followed by mass spectrometry can contribute to the definition of proteins that interfere with the complex network of the EGFR signal transduction pathway. Furthermore, the proteomic profile of cancer cell lines can contribute to the understanding of growth regulation and its modulation by tyrosine kinase inhibitors such as gefitinib. Based on the definition of differentially expressed proteins, further investigations can guide us to their physiological and pathological role and provide us

with a deeper understanding of resistance mechanisms against anticancer drugs.

Conflict of interest statement

None declared.

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