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Genome-wide gene expression analysis of chemoresistant pulmonary carcinoid cells

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Purpose: Carcinoids are highly chemoresistant tumors associated with a dismal prognosis. This study involved a comparison of the genome-wide gene expression pattern of a chemoresistant and a chemosensitive pulmonary carcinoid cell line to reveal factors that contribute to the resistant phenotype.

Materials and methods: Gene expression of UMC-11 chemoresistant carcinoid cells as assessed by 32 K microarray was compared with H835 chemosensitive carcinoid cells, and the genes that were differentially expressed and expected to be related to chemoresistance were selected.

Results: Drug-resistant UMC-11 cells exhibited increased expression of transcripts known to confer resistance to different cytostatics such as P-glycoprotein, multidrug resistance-associated proteins 2 and 3, effectors of the glutathione detoxification and xenobiotics degradation pathways, and ion transporters including Na^+/K^+-adenosine triphosphatase. In addition, enhanced transcription of several S100 proteins, capable of suppressing apoptosis, regulation of topoisomerase I (topo I) expression by antisense transcripts from *TOPO1* pseudogenes, and alterations of the cytoskeleton seem to contribute to the multidrug-resistant phenotype. A multitude of epidermal growth factor (EGF)-related and neuropeptide growth factors, overexpression of proteases, and appearance of aerobic glycolytic metabolism complement the malignant phenotype of the UMC-11 cells.

Conclusion: The multidrug-resistant phenotype of the UMC-11 pulmonary carcinoid cell line seems to be mediated by classical efflux pumps, drug metabolization or conjugation systems, and, possibly, modulation of apoptotic cell death by S100 proteins and topo I expression by pseudogene transcripts. Autocrine or paracrine stimulation by a host of EGF-related and neuropeptide growth factors, as well as high metastatic potency indicated by increased expression of components of aerobic glycolysis and proteolytic enzymes, may furthermore account for the failure of therapeutic interventions.

Keywords: neuroendocrine tumor, drug resistance, microarray, drug transporter, apoptosis

Introduction

Pulmonary carcinoid tumors are considered low-grade malignant neoplasms of the neuroendocrine cells, originating from the Kulchitsky cells of the bronchial mucosa layer, and represent about 1%–5% of all lung tumors.[1–3] Ninety percent of these tumors termed *typical carcinoids* are well differentiated and characterized by a small degree of mitosis, pleomorphism, and necrosis.[4,5] The remaining 10% of the tumors with increased mitotic activity, nuclear pleomorphism, and cellular irregularity are classified as aggressive *atypical carcinoids*.[6,7] The patients with lung carcinoids can be cured by surgical resection, but a fraction of tumors gives rise to widespread metastasis within

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several years after primary treatment.[8,9] In contrast to the more indolent, well-differentiated neuroendocrine tumors exhibiting metastases in less than 15% of cases and revealing a 5-year survival rate of more than 90%, well-differentiated neuroendocrine carcinomas are more aggressive, develop metastases in 30%–50% of cases, and yield a 5-year survival rate between 40% and 60%.[1,2,10] Almost all recurrences of either typical or atypical carcinoids involve distant sites. Finally, patients with malignant carcinoids show a 5-year survival rate of about 20% and a median survival of 2 years in the presence of liver metastases.[11] Medical treatment of metastatic disease includes somatostatin (SST) analogs, α-interferons, and chemotherapy.[1,12]

Basically, chemotherapy can be considered for patients with carcinoids progredient under noncytotoxic therapy, provided that they have significant symptoms and poor prognosis.[13,14] Clinical trials demonstrated that the response rates to single-agent chemotherapy limited to approximately 20%, and for multiagent chemotherapy, the response rates are invariably less than 40%. Moreover, these responses are frequently short-lived and rarely translate to prolonged survival. In contrast, responses of rapidly proliferating, poorly differentiated (atypical or small cell-like) carcinoid tumors to chemotherapy are high but the duration of response is extremely short. In these studies, doxorubicin, 5-fluorouracil (5-FU), dacarbazine, streptozotocin, cyclophosphamide, cisplatin, and etoposide were used either as single agents or in combinations, but eventually were not recommended for clinical routine practice due to low response rates.[1,3,11,15] The situation is complicated by the fact that these clinical trials are usually small studies with variable criteria for inclusion and study design due to the rarity of these tumors. Little is known about the mechanisms contributing to chemoresistance of carcinoids or other well-differentiated neuroendocrine tumors. Screening of chemosensitivies of UMC-11, H727, and H835 pulmonary carcinoid cell lines in the laboratory revealed the resistance of UMC-11 to 9 of 14 chemotherapeutics, whereas H727 and H835 were resistant to 4 of 14 and 5 of 14 of the drugs, respectively.[16] Because doubling times were 20.8 hours for H727, 27.0 hours for UMC-11, and 35.7 hours for H835 cells, the more rapidly proliferating and, therefore, probably more chemosensitive H727 cell line was not used in the following gene expression analysis.[16] The aim of this study was to compare the transcriptome of the highly chemoresistant UMC-11 with the sensitive H835 cell line to delineate candidate genes possibly conferring multidrug resistance to pulmonary carcinoids.

Materials and methods

Chemicals and cell lines

Unless otherwise noted, all chemicals and solutions were obtained from Sigma-Aldrich (St. Louis, MO). UMC-11 and H835 pulmonary carcinoid cell lines were purchased from the American Tissue Culture Collection (Manassas, VA). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Seromed, Berlin, Germany), 4 mM glutamine, and antibiotics. UMC-11 was subcultured by trypsinization (2.5% trypsin in EDTA solution; Boehringer Mannheim, Germany), and H835 cells growing in suspension were maintained by replacement of the medium and dispersed by trypsin treatment.

Cell proliferation assay

Cells were harvested, counted, and distributed into the wells of flat-bottomed 96-well microtiter plates at a density of 1×10^4 cells/well in 100 µL medium. 100 µL of appropriate dilutions of test compounds was added to each well, and the plates were incubated under tissue culture conditions for 4 days. Stock solutions of the compounds were prepared in either 70% ethanol or dimethyl sulfoxide and diluted more than 100-fold for use in chemosensitivity assays. Solvent-control wells were included in all tests. Dose–response curves were obtained by the assessment of cell growth at 2-fold drug dilutions in triplicate and used for the calculation of the half maximal inhibitory concentration (IC_{50}) values. Cell proliferation was quantified using a modified tetrazolium dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (EZ4U; Biomedica, Vienna, Austria).

Genome-wide gene expression analysis

Lysates of 30×10^6 cells (extraction buffer: 4 M guanidine isothiocyanate, 0.5% sodium *N*-lauroyl sarcosinate, 10 mM EDTA, 5 mM sodium citrate, and 100 µM β-mercaptoethanol; 30 minutes, 4°C) were added to cesium trifluoroacetate and centrifuged (46,000 rpm, 15°C, 20 h). Supernatant containing DNA was removed and RNA was precipitated with ice-cold 96% ethanol. Pellets were washed and, following removal of ethanol, resuspended in sterile water. RNA content was measured photometrically.

Gene expression analysis was performed using the Applied Biosystems (ABI) Human Genome Survey Microarray V2.0 (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Therefore, 2–5 µg mRNA (20–50 µg total RNA) was reversely transcribed (RT) to first-strand cDNA (MyCycler thermocycler; Bio-Rad, Hercules, CA). The RT mixture was labeled on ice and

purified according to the manufacturer's instructions for the Applied Biosystems 1700 RT Labeling Kit v002 (Applied Biosystems). Hybridization of cDNA and microarray analysis (Applied Biosystems 1700) were carried out following the manufacturer's chemiluminescence detection kit protocol. Data for each cell line (n = 2) were filtered, normalized, and log2-transformed before further processing was done using Microsoft Excel software (Microsoft, Redmond, WA) or Statistical Analysis of Microarray (false discovery rate of 10%; Stanford University, Stanford, CA). ABI 1700 gene identities can be accessed via the Panther classification system (www.pantherdb.org).

Results

We previously reported the following IC_{50} values (IC_{50} UMC-11/IC_{50} H835, mean ± standard deviation) for a range of drugs that were obtained for UMC-11 and H835 cells in chemosensitivity assays: vinblastine (ng/mL), 2 ± 0.35/2 ± 0.3; taxol (ng/mL), 10 ± 4.4/9 ± 3.9; camptothecin (nM), 18 ± 5.0/6.5 ± 4.3; 5-FU (μM), 30 ± 4.6/45 ± 8.7; doxorubicin (nM), 370 ± 72/73 ± 2.4; gemcitabine (nM), 833 ± 103/30.0 ± 20; tamoxifen (μM), 9 ± 3.3/7 ± 2.4; cisplatin (μM), 33 ± 15/33 ± 7.0; oxaliplatin (μM), 5 ± 0.25/6 ± 0.51; carboplatin (μM), 36 ± 7.1/36 ± 9.9; mitomycin (ng/mL), 156 ± 15/35 ± 9.0; streptozotocin (μg/mL), 180 ± 33/60 ± 19; etoposide (μg/mL), 45 ± 12.3/0.25 ± 0.02; and dacarbazine (μg/mL), 4 ± 2.3/16 ± 3.9. Thus, UMC-11 cells were resistant to taxol, camptothecin, 5-FU, doxorubicin, gemcitabine, tamoxifen, cisplatin, streptozotocin, and etoposide (IC_{50} exceeding peak plasma concentration).[16]

Genome-wide expression analysis was performed for chemoresistant UMC-11 and chemosensitive H835 pulmonary carcinoid cells, and the genes that were significantly overexpressed more than 4-fold in the former cell line compared with the latter were grouped according to their cellular functions and pathways (Table 1). Genes were furthermore selected in regard to their possible relation with the resistant phenotype of the UMC-11 cells. The low number of genes with lower expression in UMC-11 compared with H835 cells was not included in this analysis because of the absence of transcripts with a connection to chemoresistance. After filtering of the 1,520 transcripts that were overexpressed more than 4-fold in UMC-11 compared with H835 carcinoid cells, 386 were annotated to the corresponding genes, and 102 of these are expected to be involved in proliferation, metastasis, and drug resistance of the carcinoid cells, as listed in Table 1.

In UMC-11 cells, enzymes mainly involved in glucose and energy metabolism that were preferentially expressed included lactate dehydrogenase (*LDH/LDHB*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), hexokinase 1 (*HK1*), citrate synthetase (*CS*), and pyruvate dehydrogenase kinase 4 (*PDK4*), indicating differences in glucose uptake and utilization between the 2 cell lines. Increased expression of hypoxia-inducible gene domain family member 1A (*HIG1/HIGD1A*) and aryl hydrocarbon receptor nuclear translocator 2 (*ARNT2*) together with the changes in regard to glucose metabolization pointed to the presence of an increased rate of aerobic glycolysis (Warburg effect) in UMC-11 cells. Furthermore, in these cells, a number of proteases and protease inhibitors were highly expressed, including serine proteases 1–3 (*PRSS1-3*), serine protease inhibitors (*SERPINs A2, E2, B6,* and *A10*), cathepsin L (*CTSL*), matrix metallopeptidase 13 (*MMP13*, identical to collagenase 3), and the corresponding tissue inhibitors of metalloproteinase 1 and 3 (*TIMP1* and *3*). Hypoxia is known to select for an aggressive cancer phenotype characterized by an increased capacity to infiltrate tissues.

In addition, UMC-11 cells showed higher expression of a large number of membrane transporters compared with H835 cells, thus comprising Na^+/K^+-adenosine triphosphatase ([ATPase], *ATP1B1*), the FXYD regulatory subunit 2 (*FXYD2*), and ion pumps regulating intracellular Ca^{2+} concentration (*ATP2A2, ATP2B1,* and *ATP2C2*). The subfamily members of ATP-binding cassette (ABC) transporters (*ABCB1, C2,* and *C3*) resemble the classical resistance proteins P-glycoprotein (P-gp) and multidrug resistance-associated proteins (MRPs) 2 and 3. Furthermore, the 2 solute carrier organic anion transporters (*SLCO1A2* and *SLCO2A1*), shuttling bile acids, prostaglandins (PGs), and other anions, and a number of solute carriers transporting aspartate/glutamate (*SLC1A6*), biogenic amines (*SLC18A1*), anions (*SLC4A2*), sodium (*SLC4A11*), potassium chloride (*SLC12A7*), sodium phosphate (*SLC20A2*), and guanosine diphosphate-fucose (*SLC35C1*) were found to be upregulated.

Moreover, a large number of growth factors and receptors were overexpressed in UMC-11 compared with H835 cells, including members of the epidermal growth factor (EGF) family, insulin-like growth factor (IGF) family, neuropeptides, and others. In particular, EGF receptor (*EGFR*), v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (*ERBB3*), transforming growth factors (TGFα, β1, and β2), epiregulin (*EREG*), and amphiregulin (*AREG*) constitute a growth regulatory system, with heat shock protein 90 (*Hsp90*) and sprouty 2 (*SPRY2*) as modulators. A second independent growth factor pathway was provided by IGF2 and regulatory binding proteins (*IGFBP1, 3, 5, 6,* and

Table 1 Relative mRNA expression of selected genes in UMC-11 and H835 carcinoid cell lines

Gene symbol	Gene ID ABI 1700	Ratio of expression UMC-11/H835
Metabolism		
LDHB	167986	280
LDH	208956	55
GAPDH	236913	9
HK1	178010	8
CS	200851	6
PDK4	101060	12
Hypoxia		
HIG1	127432	12
HIGD1A	139216	14
ARNT2	205730	4
Proteases/protease inhibitors		
PRSS1	111365	2,636
PRSS2	109205	5,576
PRSS3	154524	1,861
Serpin A2	192373	11
Serpin E2	210342	13
Serpin B6	113902	5
Serpin A10	101912	18
CTSL	172735	32
MMP13	162909	20
TIMP1	134692	1,208
TIMP3	179538	57
Membrane transporters		
ATP1B1	199586	7
ATP2A2	142525	4
ATP2B1	102967	6
ATP2C2	146652	51
FXYD2	146341	84
FXYD3	126166	23
ABCB1	182279	44
ABCC2	115883	26
ABCC3	109767	41
ABCD1	165343	5
ABCG1	210662	4
SLCO1A2	192702	4
SLCO2A1	178284	33
SLC1A6	112712	916
SLC18A1	127370	27
SLC4A2	164313	4
SLC4A11	124838	16
SLC12A7	187225	6
SLC20A2	132288	5
SLC35C1	207403	8
Growth factors		
EGFR	136952	64
ERBB3	157084	22
TGFA	180395	17
TGFB1	170749	21
TGFB3	142790	8
EREG	100064	201
AREG	123143	147
Hsp90	148821	4
SPRY2	207231	33
IGFII	129922	814
IGFBP1	202509	42
IGFBP3	104923	20
IGFBP5	170211	6
IGFBP6	150353	23
IGF2BP2	147646	24
IL-8	176899	115
CALCB	180834	6,969
VIP	144138	552
NTS	160562	93
VGF	174736	36
NPW	164302	6
AGT	196523	5
SST	157922	887
FGF12	138024	13
ADCY2	193946	618
STIM2	205035	4
S100 Ca^{2+}-binding proteins		
A2	192373	11
A4	155850	170
A6	154326	87
A10	213843	185
A11P	187317	178
P	165541	254
A14	185644	36
A16	150790	432
Cytoskeleton		
Keratin I	231137	458
Keratin II	178654	103
KRT7	207298	38
KRT8	111916	14
KRT18	114812	171
KRT19	113888	80
CDH1	130505	300
VIL1	191456	264
CLDN4	156519	21
CLDN7	163619	34
CLDN20	159987	74
TUBB3	203100	10
TMSB4X	141609	4
Sulfur metabolism		
GSTT1	163516	4
GSTP1	214807	225
GRX	188218	57
ARSE/D	139887	52
SULF2	116303	43
GSTO2	177000	41
GLRX	188218	57
TST	203712	8
Detoxification		
AHR	166278	39
CYP26B1	182081	28
CYP2B6	209472	19
ALDH7 A1	219563	30
AKR7 A3	179819	7
Topoisomerase I		
TOP1	179728	7
TOPP1	115448	5
TOPP2	236556	5

IGF2BP2), supplemented by a number of neuropeptides, namely vasointestinal peptide (*VIP*), neurotensin (*NTS*), *VGF* (VGF nerve growth factor inducible), and neuropeptide W (*NPW*). Interleukin-8 (IL-8), calcitonin-related polypeptide β (*CALCB*), angiotensin (*AGT*), and fibroblast growth factor 12 (*FGF12*) may in addition be involved in the regulation of cell proliferation, with SST as inhibitor. Increased overexpression of adenylate cyclase (*ADCY2*) pointed to an important role of this enzyme in signal transduction pathways of the growth stimulators.

S100 proteins regulate intracellular processes, such as cell growth and motility, cell cycle regulation, transcription, and differentiation. Twenty members have been identified so far, and altogether, S100 proteins represent the largest subgroup of the EF-hand Ca^{2+}-binding protein family. A unique feature of these proteins is that individual members are localized in specific cellular compartments from where some are able to relocate upon Ca^{2+} activation, thus transducing the Ca^{2+} signal temporally and spacially by interacting with different targets highly specific for each S100 protein. Some of the S100 Ca^{2+}-binding proteins overexpressed in the UMC-11 cells, namely *A2, A4, A6, A10, P, A14,* and *A16*, seem to have important functions in Ca^{2+} homeostasis, growth, and apoptosis. Differences in the expression of cytoskeleton constituents comprised type I keratins (*KRT18* and *KRT19*), type II keratins (*KRT7* and *KRT8*), cadherin 1 (*CDH1*), villin 1 (*VIL1*), claudins (*CLDN4, 7*, and *20*), tubulin β3 (*TUBB3*), thymosin β4, X-linked chromosome (*TMSB4X*), and stromal interaction molecule 2 (*STIM2*).

Finally, detoxification of xenobiotics may be mediated by conjugation to sulphur-containing compounds, and the corresponding enzymes comprising glutathione *S*-transferase θ1 (*GSTT1*), glutathione *S*-transferase π1 (*GSTP1*), glutaredoxin (*GRX*), arylsulfatase (*ARSE*/*D*/*SULF2*), glutathione *S*-transferase Ω2 (*GSTO2*), and thiosulfatase (*TST*) were found to be overexpressed in UMC-11 cells. Other mechanisms for the metabolization of drugs in UMC-11 cells included the cytochrome P450 (CYP) oxidoreductases (*CYP26B* and *CYP12B6*) that are regulated by the consistently overexpressed aryl hydrocarbon receptor (AHR), aldehyde dehydrogenase 7A1 (*ALDH7A1*), and aldo-keto reductase 7A3 (*AKR7A3*).

Topoisomerases are important targets of a range of cytotoxic drugs, in particular (*topoI*) for camptothecin derivatives like irinotecan and topotecan. Although *TOP 1* expression was elevated in UMC-11 cells, increased amounts of antisense transcripts stemming from *TOPP1* and *TOPP2* are expected to downregulate *TOP1* to induce drug resistance.

Discussion

Neuroendocrine lung tumors are highly refractory to chemotherapy, but the detailed mechanisms resulting in chemoresistance have not been fully characterized so far.[11] In contrast to the typical carcinoids, where multidrug resistance (MDR) is mediated by P-gp but not MRP, the atypical carcinoids exhibit cellular detoxification by P-gp, MRP, and GST-π expression.[17] Both Bcl-2 and p53 proteins appear unchanged in carcinoids, suggesting that the apoptotic capacity conferred by these two gene products is not involved in chemoresistance. The chemoresistance of typical carcinoids was questioned, in general, based on their sensitivities observed in *in vitro* tests using these well-differentiated tumors.[13] We recently compared the chemosensitivity profiles of the three carcinoid cell lines UMC-11, H727, and H835 and demonstrated marked chemoresistance to a wide range of cytotoxic drugs in UMC-11 cells in contrast to the other two chemosensitive cell lines.[16] In this study, genome-wide gene expression analysis was used to search for candidate genes overexpressed in UMC-11 cells compared with H835 cells, and that would possibly hold the potential to confer chemoresistance.

Expression of hexokinase (HK1), an enzyme that traps glucose inside the cell by catalyzing phosphorylation to glucose 6-phosphate, and thus, maintains the downhill concentration gradient permitting facilitated glucose transport into cells for utilization, was higher in UMC-11 than in H835 cells.[18] GAPDH catalyzes the conversion of glyceraldehyde 3-phosphate to D-glycerate 1,3-bisphosphate during the sixth step of glycolysis. LDH/LDHB catalyzes the conversion of L-lactate and nicotinamide adenine dinucleotide (NAD) to pyruvate and NADH in the final step of anaerobic glycolysis. This enzyme normally converts pyruvate, the final product of glycolysis, to lactate under hypoxic conditions. *PDK4* is a member of the PDK/BCKDK protein kinase family and encodes a mitochondrial protein with a histidine kinase domain.[19] This protein inhibits the PD complex (PDC) by phosphorylating one subunit, which contributes to the regulation of glucose metabolism. The PDC converts pyruvate to acetyl coenzyme A (acetyl-CoA) by decarboxylation. Acetyl-CoA may then enter into the citric acid cycle for cellular respiration, thereby the glycolytic metabolic pathway is linked to the citric acid cycle and energy released in the form of NADH PD. In summary, high expression of these enzymes may indicate a shift of the cellular metabolism to increased uptake and catabolization of glucose.

Tumor cells exhibit aerobic glycolysis frequently (Warburg effect), and according to our results, the increased expression of a number of relevant genes corroborate

this metabolic shift. Unlike in H835 cells, *HIGD1A* is overexpressed in UMC-11 cells. *HIG1* was found as a novel gene, with unknown function induced by hypoxia and glucose deprivation in human cervical epithelial cells *in vitro*.[20] Hypoxia-induced transcription of the HIF-target genes *HIGD1A*, egl nine homolog 1 (*EGLN1*), Bcl2-interacting-protein 3 (*BNIP3*), and phosphofructokinase 1 is independent of the CH1 domain of CBP/p300, whereas stanniocalcin 1 and *SLC2A1* (glucose transporter 1) were moderately affected by the alteration of this transcriptional coactivator.[21] Similarly, *EGLN1, HIG1*, and prolyl 4-hydroxylase are induced by treatment with nickel.[22] In good agreement with *HIGD1A* expression, abundant transcripts of *ARNT2*, a member of the basic-helix-loop-helix-Per-Arnt-Sim (bHLH-PAS) superfamily of transcription factors that was observed in UMC-11 cells, heterodimerize with HIF-1 in the nucleus and, thus, promote expression of oxygen-responsive genes. In contrast to HK1, GAPDH is known to be induced by hypoxia or nickel.[23,24] Therefore, UMC-11 cells clearly showed signs of aerobic glycolysis and induction of hypoxia-induced genes under aerobic conditions.[25]

Most cancers exhibit elevated protease levels that contribute to certain aspects of tumor behavior in regard to growth, metastasis, and angiogenesis. Increased expression of the CTSs of the cysteine protease family, such as CTSL, correlates with invasion and migration of highly metastatic B16 melanoma cells.[26] Trypsin 1 and 2, serine protease 3 (*PRSS1, 2*, and *3*), and *MMP13* were preferentially expressed in UMC-11 but not in H835 cells. Trypsin is implicated in colorectal carcinogenesis and promotes growth and dissemination of various cancers.[27] Furthermore, trypsin expression is an indicator of poor prognosis and shorter disease-free survival of colorectal cancer patients. Trypsin seems to act either directly by the digestion of type I collagen or indirectly by the activation of other proteinase cascades like MMPs. Stimulated by trypsin, both MMPs and protease-activated receptor 2 may activate the mitogen-activated protein kinase–extracellular signal-regulated protein kinase (MAPK-ERK) pathway through EGFR. MMPs may play an important role in both transformation from adenoma to carcinoma and initiation of invasion and metastasis. Upregulation of MMP13 at the tumor-bone interface is crucial for tumor-induced osteolysis, which suggests that MMP13 is a potential therapeutic target for breast cancer bone metastasis.[28] Tumor cell proteases are under tight regulation by proteinase inhibitors like TIMP2 and 3, both acting on MMP13.[29] Moreover, the highly expressed serpins (serine protease inhibitors) A1, A10, E2, and F1 are proteins with similar structures that were first identified as inhibitors.[30] *SERPINA10* was one of the six novel marker genes for neuroendocrine carcinoma cells comprising paraneoplastic antigen *MA2* and testican 1 precursor (*SPOCK1*) among others.[31] Overexpression of these proteases in conjunction with their regulatory inhibitors in UMC-11 cells pointed to a highly metastatic and aggressive phenotype.

The solute carrier, human organic anion transporting polypeptide *SLCO1A2* is highly expressed in the intestine, kidney, cholangiocytes, and the blood–brain barrier.[32] This localization suggests that the transporter may be crucial for the distribution of clinically important drugs, besides shuttling bile acids. *SLCO2A1* transports PGs like PGE2 that was identified as the principal prostanoid promoting cell growth and survival in colorectal tumors by increasing Bcl-2 expression, which promotes cell survival under hypoxic conditions.[33] The other solute carriers overexpressed in UMC-11 cells are involved in the maintainance of ion homeostasis (Na^+, K^+, H^+, and others), with no clear relation to chemoresistance.

MDR of tumor cells against a whole group of cytotoxic compounds is mediated by ABC efflux pumps.[34] P-gp (*ABCB1*), encoded by the *MDR1* gene, was the first such transporter detected. Determination of *MDR1* mRNA in human cancers revealed elevated expression in untreated, intrinsically drug-resistant tumors, including those derived from carcinoid tumors.[35] Further studies resulted in the characterization of the MRPs in some drug-selected cancer cell lines.[36] UMC-11 cells expressed *ABCC2* (MRP2) and *ABCC3* (MRP3) at higher levels than the drug-sensitive H835 cell line. MRPs appear to mediate glutathione (GSH) homeostasis and the efflux of oxidized GSH derivatives (eg, glutathione disulfide, *S*-nitrosoglutathione, and glutathione-metal complexes) and other glutathione *S*-conjugates.[37] Among the many processes, apoptosis, cell proliferation, and differentiation are influenced by GSH transporters. MRP gene expression was detected in almost all lung cancer cell lines, including carcinoids and normal lung tissue.[36] Furthermore, *ABCG1* and *ABCD1* are involved in the transport of lipids. In conclusion, MDR in UMC-11 cells seems to be partially effectuated by the classical ABC drug efflux pumps.

UMC-11 cells exhibited higher expression of *ATP1B1* encoding the Na^+/K^+-ATPase that exchanges three intracellular sodium for two extracellular potassium ions and is upregulated by cyclic adenosine monophosphate (cAMP). It serves as a signal transducer, regulates cell adhesion, and its aberrant expression and activity are implicated in the development and progression of different cancers.[38] Inhibition of this pump by cardiac glycosides reduces tumor metastasis, which

is possibly due to the sensitization to anoikis.[39] Altered ion gradients were also proposed as a potential cause of resistance to chemotherapy.[40] Furthermore, inhibition of Na^+/K^+-ATPase markedly reduces intracellular cisplatin accumulation.[41] A hemisynthetic cardenolide shows high anticancer activity in cells expressing diverse forms of MDR, which is either conferred by inherent overexpression of selected drug transporter proteins or induced by a range of chemotherapeutic agents.[42] Ion transport by Na^+/K^+-ATPase is modulated by a regulatory subunit belonging to the FXYD protein family.[43] UMC-11 cells showed high levels of *FXYD2* and *FXYD3* constituting the Na^+/K^+-ATPase γ-subunit and an ion pump regulator, highly expressed in breast cancers and responsible for cancer cell proliferation, respectively.[44] The other ion pumps overexpressed in UMC-11 cells namely *ATP2A2, B1*, and *C2* are responsible for the maintainance of cytoplasmic Ca^{2+} homeostasis.

Growth factors and their cognate receptors do not confer chemoresistance, but since they determine the proliferation rate, they constitute interesting targets for chemotherapy. The EGF/EGFR and ERBB3 growth factor system, as found overexpressed in UMC-11 cells, comprises a range of receptors and mitogenic factors including TGFα and others. The coexpressed factors TGFβ1 and β2 are involved in the regulation of many physiological processes like cell growth, cell differentiation, and extracellular matrix production among others.[45] Moreover, TGFβ may function as tumor suppressor or promotor depending on the type, differentiation state, and physiological characteristics of target cells.[46] The ErbB ligand epiregulin, found in UMC-11 cells, is highly expressed in non-small cell lung carcinoma (NSCLC) and correlates with nodal metastasis and a shorter duration of survival.[47] The related and coexpressed EGFR/TGFR ligand, amphiregulin, was found to predict NSCLC resistance to gefitinib and to suppress apoptosis by sequestration of BAX in the cytoplasm.[48] EGFR and activated EGFR (p-EGFR) were expressed in both well-differentiated gastrointestinal carcinoids and pancreatic endocrine tumors in primary and metastatic specimens with a poor prognosis.[49] The role of EGFR was studied in pulmonary typical and atypical carcinoid tumors.[50] Analysis showed that approximately half of typical carcinoids and one-third of atypical carcinoids produce EGFR, and all of the tumors exhibit moderate to intense staining for ErbB3 and ErbB4 but lack expression of ErbB2. High expression of the chaperone Hsp90 stabilizes oncogenic kinases and mutant EGFR driving proliferation of lung cancer cells.[51] It was recently demonstrated that mutant EGFR is a Hsp90 target. In addition, SPRY1 seems to be operative in the regulation of EGFR signaling.[52]

Another growth factor system frequently overexpressed in cancers is represented by the IGFs. IGF2 and a number of associated regulatory IGF-binding proteins (*IGFBP1, 3, 5, 6,* and *IGF2BP1/2*) were elevated in UMC-11 cells. Expression of *IGFBP2* along with low amounts of *IGFBP1* was found in all carcinoid samples in association with varying occurrences of *IGF1R, IGF2R*, and *IGFBP6*.[53,54] CALCB was reported to play an important role in the proliferation of various types of epithelial and endothelial cells.[55] In an *in vitro* study, calcitonin-gene related peptide increased the proliferation of A549 alveolar epithelial cells in a dose-dependent and time-dependent manner.[56] IL-8 is a chemokine and angiogenic factor that also functions as an autocrine growth factor in several human cancers. In lung cancer, all NSCLC cell lines produced IL-8, in contrast to low levels of IL-8 in SCLC cell lines despite expression of the IL-8 receptors CXCR1 and CXCR2.[57] FGF12 is a FGF-homologous factor, which interacts with the intracellular kinase scaffold protein islet brain-2 and voltage-gated sodium channels.[58] UMC-11 cells produce AGT that may influence tumor growth and metastasis in a tissue-specific and tumor-specific manner.[59] In contrast, SST released by UMC-11 cells counteracts signaling of many other growth factors that contribute to proliferation, such as prolactin, IGF, TGFα and β, platelet-derived growth factor, EGF, and vascular endothelial growth factor (VEGF).

Because carcinoids resemble neuroendocrine tumors, the high expression of neuropeptides comprising VIP, NTS, VGF, and NPW by UMC-11 cells is expected to contribute significantly to autocrine growth stimulation. VIP causes increased proliferation of human breast and lung cancer cells *in vitro*.[60] It binds to cancer cells with high affinity and increases cAMP and gene expression of c-fos, c-jun, c-myc, and VEGF. SCLC, which is a neuroendocrine cancer, produces and secretes gastrin releasing peptide (GRP), NTS, and adrenomedullin (AM) as autocrine growth factors.[61] Most of these neuropeptides are detectable in serum samples of carcinoid patients.[62] GRP, NTS, and AM bind to G-protein-coupled receptors triggering increased phosphatidylinositol (PI) turnover or elevation of cAMP in SCLC cells. Addition of GRP, NTS, or AM to SCLC cells causes altered expression of nuclear oncogenes like c-fos and stimulation of growth.[63] Analysis of peptides secreted by the large-cell neuroendocrine carcinoma cell line revealed 2 fragments that were demonstrated to be parts of VGF, which is usually expressed in nerve cells or neuroendocrine cells.[64] Reverse transcription–polymerase chain reaction of lung cancer cell lines showed that VGF mRNA was expressed only in neuroendocrine carcinoma-derived cells. Neuropeptide

B (NPB) and NPW are the endogenous ligands of the 2 Gprotein-coupled receptors GPR7 and GPR8 and activate protein kinase A and protein kinase C (PKC) signaling.[65] NPB and NPW were reported to stimulate tyrosine kinase and MAPK activities, mediating the proliferative and anti-apoptotic effects of NPB and NPW.

Cell type–specific differences exist among the various signal transduction pathways, comprising cAMP, hydrolysis of PI mobilization of intracellular calcium, and tyrosine phosphorylation, and, moreover, different receptors for the same trigger may be linked to different signal transduction pathways.[66,67] Basically, the mitogenic signal of neuropeptides is transmitted into the cell via specific receptors that couple to heterotrimeric G proteins. Subsequent activation of phospholipase C-β influences the activation of PKC and the elevation of intracellular calcium. The antiproliferative effect of the Ca^{2+}-channel blocker verapamil was investigated *in vitro* on 3 human lung cancer cell lines.[68] Verapamil inhibited cell proliferation in the neuroendocrine cancer cell line H727 in the nM range. STIM2 overexpressed in UMC-11 cells functions as highly sensitive Ca^{2+} sensor in the endoplasmic reticulum and regulates the ion concentration in conjunction with different pumps. Compared with H835, UMC-11 cells showed high expression of adenylate cyclase, establishing cAMP as an important second messenger of growth signals in these resistant carcinoid cells. In conclusion, UMC-11 cells seem to rely on EGF-related, IGF-related, and neuropeptide growth factors for stimulation of proliferation, and this multitude of autocrine and paracrine loops is expected to impede antiproliferative therapy by kinase inhibitors.

Overexpression of a large number of S100 Ca^{2+}-binding proteins in UMC-11 cells pointed to their important functions in cell biology and possibly drug resistance of carcinoids. Intracellular Ca^{2+} seems to be permanently elevated in UMC-11 cells in response to continuous stimulation by growth factors. Downregulation of proapoptotic genes such as serine/threonine kinase 17a and *BNIP3* in association with *S100P* was reported to be linked to oxaliplatin resistance in the THC8307/L-OHP colon cancer cell line, and 8.7-fold overexpression of S100 protein family members was demonstrated in proteome analysis of cisplatin-resistant ovarian cancer cells.[69,70] Furthermore, pancreatic ductal adenocarcinoma showed a correlation between decreased expression of *BNIP3* and chemoresistance through repression by *S100A2* and *S100A4*.[71] In support of these findings, *S100A4* expression was a predictor of poor prognosis for T1N0M0 breast cancer patients.[72,73] In conclusion, the expression of a number of S100 proteins may contribute to the broad chemoresistance of carcinoid cells by downregulation of proapoptotic factors.

Numerous members of the cytoskeleton are preferentially expressed in UMC-11 vs H835 cells, including type I and II cytokeratins, CLDNs, VIL, CDH1, TUBB3, and thymosin β4. Most of these components may reflect differences in the histological subtypes of UMC-11 and H835 cells; however, especially TUBB3 overexpression represents a marker of resistance to microtubule-targeting drugs *in vitro*, *in vivo*, and clinically for many tumors, including breast cancer, and thymosin β4 proves to be a gene associated with response to therapy in ovarian cancer.[74,75]

Sulfur-containing molecules such as cysteine, methionine, GSH, metallothioneins, and albumin bind platinum-based compounds and reduce therapeutic efficacy at the level of uptake, excretion, resistance, and toxicity.[76] Studies showed that variability in survival can in part be explained by polymorphisms in genes encoding proteins involved in degradation of drugs. Most importantly, such polymorphisms of drug-metabolizing enzymes and transporters demonstrated to influence survival after cancer treatment compass genes of the phase II detoxification enzymes, GSTs.[77] For example, the *GSTM1*-deficient and *GSTT1*-deficient genotypes have a clear association with longer overall survival in patients treated with respective substrates, such as alkylating agents and platinum compounds. Likewise, genetic polymorphisms of *GSTP1* and *GSTA1* are also linked with increased overall survival in patients with different malignancies. Levels of thioredoxin (TRX) and GRX were elevated in pancreatic ductal carcinoma tissues compared with pancreatic cystadenocarcinoma or normal pancreatic tissue. Furthermore, cisplatin-resistant subclones of HeLa cells had higher expression of TRX and GRX compared with the parental cells.[78]

AHR encodes a ligand-activated transcription factor binding to the xenobiotic response element-containing promoter region that is involved in the regulation of biological responses to planar aromatic hydrocarbons. This receptor has been shown to regulate levels of xenobiotic-metabolizing enzymes like CYP, of which CYP26B1 and 2B6 were comparatively overexpressed in UMC-11 cells.[79] Human ALDH7A1 protects against hyperosmotic stress through generation of osmolytes and metabolization of toxic aldehydes.[80] Human ALDH7A1 expression in Chinese hamster ovary cells attenuated osmotic stress-induced apoptosis caused by increased extracellular concentrations of sucrose or sodium chloride. AKRs are phase I drug-metabolizing enzymes for a variety of carbonyl-containing drugs and are implicated in cancer chemotherapeutic drug resistance. They are

stress-regulated genes and play a pivotal role in the cellular response to osmotic, electrophilic, and oxidative stress. The 10 known human AKR enzymes can turnover a range of substrates, including drugs, carcinogens, and reactive aldehydes. AKRs like AKR7A3 are soluble NAD(P)H oxidoreductases that primarily reduce aldehydes and ketones to primary and secondary alcohols, respectively.[81]

Eukaryotic topo I, a DNA unwinding enzyme, is essential for several cellular functions and is the target of camptothecin derivatives like irinotecan and topotecan. However, regulation of topo I activity was not known for a long time. In an effort to identify potential regulators of *TOP1* activity, at least 2 antisense transcripts coded by *TOP1* pseudogenes were described.[82] Although the function of these *TOP1* antisense transcripts has remained unknown, recent studies of naturally occurring antisense RNA demonstrated a number of potential regulatory roles. Downregulation of topo I in carcinoids would increase resistance to camptothecin derivatives.

Conclusion

Multidrug-resistant UMC-11 cells seem to use classical drug transporters, such as P-gp and MRP, drug conjugation and metabolization systems, and possibly new mechanisms, such as suppression of apoptosis by S100 proteins and downregulation of topo I, by its own pseudogene transcripts to achieve chemoresistance. The genome-wide search for drug resistance-associated genes described here provides new evidence for possible targets to circumvent the chemoresistant phenotype of carcinoids. Functional involvement of the candidate genes found needs to be verified by experiments deleting the corresponding transcripts, and, furthermore, it remains to be investigated how far these results apply to other neuroendocrine tumors.[83]

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