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DISSERTATION

Characterization of circulating tumor cells in locally advanced squamous cell carcinoma of the head and neck.

zur Erlangung des akademischen Grades

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von

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Abstract

About one third of the patients presenting with locally advanced squamous cell carcinoma of the head and neck (SCCHN) carries a considerable risk of metastasis and related mortality, despite the applied treatment. The mechanisms regulating tumor cell dissemination and metastasis in this patient group are largely unresolved. Therefore, the frequency of circulating tumor cells (CTCs) in peripheral blood from patients with locally advanced, inoperable disease was assessed in this study. For that purpose, a flow cytometry protocol for detection of CTCs was developed. The potential of CTCs to serve as a prognostic marker in locally advanced SCCHN was determined by correlating their presence in the blood with the clinicopathologic parameters of the patients. We observed that the presence of CTCs was significantly correlated with the grade of lymph node metastasis, whereas no correlation with T stage and tumor volume was found, which suggests that CTC detection may serve as an additional prognostic marker to the N staging.

Further molecular analyses of SCCHN cell lines revealed that EGFR activation was associated with an epithelial-mesenchymal transition (EMT)-like phenotype, which was characterized by altered E-cadherin localization, high expression levels of Snail1, an elevated migratory potential and decreased sensitivity to irradiation or cetuximab monotherapy. Therefore, a highly sensitive and specific multicolour flow cytometry protocol, which allows the analysis of CTC numbers and their EGFR expression and activation, was developed. This protocol was applied for the analysis of SCCHN patient blood samples during the course of a clinical study in order to evaluate the potential role of CTC numbers and molecular profile in predicting the efficacy of different treatment regimens. We observed that the release of CTCs in the circulation and the change in their molecular profile during therapy is a dynamic process, most probably reflecting the tumor's response to the assigned treatment. Therefore, CTC analysis in the course of treatment would allow monitoring of the disease and adjustment of the cancer-specific therapy, when necessary.

Über ein Drittel der Patienten mit lokal fortgeschrittenem Plattenepithelkarzinom des Kopfes und des Halses (SCCHN) trägt ein erhebliches Risiko der Metastasebildung, trotz der Behandlung angewendet. Die Regulierungsmechanismen der Verbreitung und Metastase von Tumorzellen in dieser Patientengruppe sind weitgehend ungelöst. Aus diesem Grund wurde in dieser Studie die Häufigkeit von zirkulierenden Tumorzellen (CTCs) im peripheren Blut von Patienten mit lokal fortgeschrittenen, nicht operierbaren Tumoren ermittelt. Zu diesem Zweck wurde ein Durchflusszytometrieprotokoll zur Erkennung von CTC entwickelt. Das Potenzial der CTCs als prognostische Marker beim lokal fortgeschrittenen SCCHN wurde durch den Zusammenhang zwischen dem Auftreten dieser Zellen im Blut und den klinisch-pathologischen Parametern der Patienten bestimmt. Es wurde festgestellt, dass die Präsenz von CTC mit der Phase der Lymphknotenmetastase erheblich korreliert, hingegen keine Korrelation mit T-Stadium und Tumorvolumen konnte beobachtet werden, was darauf hindeutet, dass CTC-Erkennung als zusätzlicher Prognoseindikator zu dem N-Stadium dienen könnte.

Weitere molekulare Analysen von SCCHN Zelllinien zeigten, dass EGFR-Aktivierung mit einem epithelial-mesenchymalen Transition (EMT) Phänotyp verbunden wurde, der durch veränderte E-Cadherin-Lokalisation, hohe Expressionsniveaus von Snail1, ein erhöhtes Migrationspotenzial und verminderte Irritationsempfindlichkeit gegenüber Bestrahlung oder Cetuximab-Monotherapie charakterisiert wurde. Daher wurde ein hochempfindliches und spezielles Multicolor-Durchflusszytometrie-Protokoll entwickelt, welches die Analyse von CTC Zahlen und ihrer EGFR-Expression und Aktivierung ermöglicht. Dieses Protokoll wurde für die Analyse von Blutproben eines SCCHN Patienten im Laufe einer klinischen

Studie verwendet, um die potentielle Bedeutung von CTC Zahlen und molekularem Profil bei der Vorhersage der Wirkungskraft von verschiedenem Behandlungsregime auszuwerten. Es wurde festgestellt, dass die Freisetzung von CTCs in die Blutlaufbahn und die Veränderung ihres molekularen Profiles während der Therapie ein dynamischer Prozess ist, der höchstwahrscheinlich die Tumorreaktion auf die Behandlung spiegelt. Demzufolge würde die CTC Analyse im Verlauf der Behandlung eine Überwachung der Krankheit und die Anpassung der krebs-spezifischen Therapie, wenn notwendig, erlauben.

Introduction

Head and neck cancer comprises approximately 6% of all cancers and it is characterized by a high mortality rate with at least one third of all patients dying as a result of their disease in 5 years after their first diagnosis. Squamous cell carcinoma of the head and neck region (SCCHN) represents more than 90% of the head and neck cancers. A significant improvement in the locoregional control of this cancer has been achieved over the last decades by the introduction of new surgical techniques and radiochemotherapy regimens (1). However, this improvement in clinical management does not seem to have significantly influenced the overall survival rate. Although early-stage head and neck cancers currently have high cure rates, up to 50% of head and neck cancer patients present with locally advanced disease and a substantial proportion of these patients develop local recurrences and distant metastasis within 2 years after diagnosis (2,3). Unfortunately, the molecular mechanisms regulating such tumor cell dissemination and metastasis formation in these patients are not completely resolved and therefore circulating tumor cells (CTCs) came in the main focus of cancer research in the recent years.

The first description of CTCs in the peripheral blood of patients with solid malignancies dates from the 19th century (4). A fraction of these CTCs presumably derives from the primary tumor as one of the initial steps in the process of metastasis, whereas the remaining CTCs probably originate from metastatic cancers. Since the likely outcome of cancer patients is largely determined by the metastatic potential of the primary tumor and the number of CTCs is likely to reflect its aggressiveness, CTC detection and quantification are considered to bear great potential as a prognostic marker in oncology (5). Indeed, CTCs detected in peripheral blood of patients with solid epithelial tumors, e.g. breast (6,7), colorectal (8,9) and prostate (10,11) cancer, have been associated with increased risk of metastasis and decreased overall survival. However, only few studies revealed the presence and the count of circulating epithelial cells also in peripheral blood of SCCHN patients (12-17), especially in a nonmetastatic setting. Additionally, the prognostic role of CTCs in locally advanced SCCHN has not been well studied so far.

Moreover, CTCs analysis might serve as a “liquid biopsy”, since peripheral blood is much more easily accessible than tumor tissue. Therefore, the identification of molecules contributing to tumor cell invasion, metastasis or therapy resistance in CTCs would potentially allow identification of novel drug targets or modification of the assigned therapy. An example of a molecule which was proven to play a major role in cancer development and metastasis is the epidermal growth factor receptor (EGFR). EGFR is commonly expressed on the surface of normal epithelial cells and at high levels in a variety of epithelial cell-derived tumors including SCCHN (18). The aberrant activation of the EGFR in SCCHN triggers a series of intracellular signals ultimately leading to the proliferation of cancer cells, induction of angiogenesis and metastasis (19). These observations suggest a link between EGFR activation and the generation of circulating metastatic cells. The latter is initiated by a disruption of the cell-to-cell adhesion mechanisms in the epithelial cell layer and acquisition of cell mobility, which is hallmarked by the activation of a cellular program termed epithelial–mesenchymal transition (EMT). EGFR has been suggested by a number of studies as a potent major member of the EMT pathway, because its activation is able to induce EMT in cancer cells (20,21). Beside the role of EMT in tumor invasion and metastasis, the EMT phenotype has been shown to interfere with the response of SCCHN cells to antitumor treatment (22-25).

Based on all these observations it is likely that CTCs might also bear prognostic significance in SCCHN. Monitoring the expression and activation of molecules involved in tumor cell dissemination and treatment resistance in these cells prior to or during treatment might predict the efficacy of the assigned therapy regimen and thereby significantly contribute to the establishment of personalized treatment.

Research aims

Several approaches to detect CTCs have been described and are classified into PCR-based and cytometric methods (26). In the past, PCR-based methods have been the most widely employed techniques for CTCs detection in SCCHN patients (12-15). However, the majority of the existing CTC-detection methods do not allow their detailed phenotypic characterization. Therefore, circulating epithelial cancer cells in peripheral blood of SCCHN patients have been analyzed in this study by flow cytometry. A flow cytometry protocol for CTC detection after depletion of CD45+ blood cells with the use of immunomagnetic separation technique (27,28) has been optimized by performing spiking experiments and applied for determining the presence of CTCs in the blood of SCCHN patients with locally advanced unresectable disease before start of therapy. The presence of CTCs and their basal number were then correlated with clinical parameters such as the size of tumor burden and extent of lymph node metastasis in order to investigate their role as an independent prognostic marker in SCCHN.

Additionally, SCCHN cell lines with different EMT morphology have been analyzed for the expression and activation status of EGFR. It was then determined whether activation of EGFR interferes with their EMT phenotype, migratory potential and their sensitivity to radiotherapy and cetuximab treatment. Thus, EGFR activation was shown to be a potential biomarker relevant to SCCHN metastasis and response to treatment, which can be studied in CTCs.

In order to further phenotypically characterize CTCs, there was a need of a highly sensitive method for CTC detection in SCCHN patients. Since an additional immunomagnetic enrichment procedure could potentially have led to loss of target cells, a flow cytometry method for CTC detection was developed by which blood cells were depleted during acquisition by using predefined electronic thresholds. CTCs were afterwards analyzed by the developed flow cytometry protocol for their basal EGFR expression and its phosphorylation status.

Finally, the change in CTC numbers and phenotype was followed by analyzing peripheral blood of SCCHN patients with locally advanced, unresectable tumors during standard chemoradiotherapy or induction chemotherapy followed by radio-/anti-EGFR antibody therapy in order to monitor disease response to treatment.

Methods and Materials

Cell lines

The colorectal cancer cell line SW620 and the SCCHN cell line UT(University of Turku)-SCC-23 were used in spiking experiments as models for epithelial EpCam+cytokeratin+ CTCs and such highly expressing EGFR accordingly. The SCCHN cell lines UD (University of Düsseldorf)-SCC-4, UT-SCC-9 and FaDu were used for studying their EMT phenotype, migratory potential and their response to irradiation and cetuximab (Merck Serono, Darmstadt, Germany) treatment. All cell lines were cultured in Minimal Essential Medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum and 1x non-essential amino acids (GIBCO, Invitrogen, Darmstadt, Germany) at 37°C and 5% CO_2 in a humidified atmosphere.

Spiking of epithelial tumor cells in peripheral blood

SW620 cells were harvested by incubation with trypsin-EDTA solution (Sigma-Aldrich GmbH, Munich, Germany). Cells were washed with phosphate-buffered saline (PBS) and resuspended in PBS at a density of 10^5 cells per ml. This suspension was used for a serial dilution of the cells. An aliquot containing the needed number of tumor cells for spiking experiments was added ("spiked") to 5 ml healthy donor blood. The spiked blood samples were processed according to the different protocols for CTC enrichment and flow cytometric detection.

Sampling and processing of peripheral blood

The analysis of CTCs in peripheral blood of patients was approved by the local ethics committee. All blood samples were collected after informed patient consent. After discarding the first 2.5 ml of blood to avoid potential contamination with skin epithelial cells, peripheral blood samples (7.5 ml) were collected into heparinized tubes (BD Biosciences Europe, Heidelberg, Germany). Samples were stored at room temperature and were further processed within 24 h after blood sampling according to the protocols for CTC detection with or without CD45 depletion described below. After flow cytometry measurement, CTCs were defined as EpCAM+cytokeratin+CD45− events. A blood sample was considered CTC+ when at least one EpCAM+cytokeratin+CD45− cell was detected. The absolute numbers of CTCs per 3.75 ml blood were determined by recording all events in the aliquot.

Evaluation of CTC correlation with clinical parameters

Flow cytometric detection of CTCs after negative enrichment

Blood erythrocytes were removed using red blood cell lysis buffer (154 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA). The remaining cells were resuspended in 1 ml staining buffer [0.5% bovine serum albumin (BSA), 2 mM EDTA in PBS]. Tumor cells were afterwards enriched by depletion of the CD45+ leukocyte fraction using a magnetic bead separation technique (EasySep®, Stem Cells Technologies, Inc., Grenoble, France) according to manufactures' instructions. The remaining cell suspension was split into two fractions, each then containing half the volume of the peripheral blood sample. These two aliquots were stained with either a cocktail of specific antibodies to EpCAM [clone EBA-1, allophycocyanin (APC)-labeled, BD Biosciences] and CD45 [clone HI30, phycoerythrin (PE)-Cy™7-labeled, BD Biosciences)] or the relevant isotype control antibodies (BD Biosciences). Cells were then fixed and permeabilized with Fix&Perm Kit (An der Grub Bio Research GmbH, Wien, Austria) reagents according to the manufacturer's instructions. During the permeabilization step the cells were stained with pan-cytokeratin [clone C-11, fluorescein isothiocyanate (FITC)-labeled, Sigma-Aldrich GmbH, Munich, Germany] or the relevant isotype control (BD Biosciences). After enrichment of tumor cells from the pool of blood cells and staining, cells were washed with PBS and immediately measured by flow cytometry (FACSCanto II, BD Biosciences). The PMT voltages used for recording of the fluorescence signals from the stained cells were as follows: FITC = 330, PE-Cy7 = 470,

APC = 420. A threshold level of 10,000 for the signal of the forward scatter was applied and no fluorescence electronic thresholds were used. The absolute numbers of tumor cells were estimated by acquiring the total number of events in the analyzed sample. Data were analyzed with BD FACSDiva™ Software v6 (BD Biosciences).

Patients and controls

42 consecutive unselected patients with histologically confirmed locally advanced, inoperable SCCHN presenting at our clinical department for treatment were included in this study. Of the 42 patients, 35 (83%) were male and the most common tumor localizations were the oropharynx (40%) and oral cavity (21%). Staging was performed according to the TNM classification system. The detailed staging can be found in the corresponding publication **"The presence of circulating tumor cells (CTCs) correlates with lymph node metastasis in nonresectable squamous cell carcinoma of the head and neck region (SCCHN)"** [Ref. (29)] . At the time point of the first blood sampling, none of the patients had started definitive chemoradiation. Blood samples were processed according to the protocol for CTC detection in peripheral blood with CD45+ cell depletion described above. Blood samples from 30 healthy individuals were also included in this study.

Detection of circulating EGFR transcripts by nested RT-PCR

As an independent method for detection of CTCs, we used detection of EGFR transcripts by nested RT-PCR (30,31). For this analysis 36 of 42 cases were available. The detailed protocol for mRNA analysis, including the primer sequences and reagents used, is described in [Ref. (29)].

Volumetric measurements

Delineation and volumetric measurements of the primary tumor and the affected lymph nodes (gross tumor volume, GTV) were done on all axial CT-slices, with a slice thickness of 3.75 mm, using the Eclipse Treatment Planning Software (version 8.6, Varian, Palo Alto, CA) or the iPlan RT Image 4.1 Software (BrainLAB AG, Germany).

Statistical analysis

The correlation between the CTC+ cases, detected by flow cytometry or RT-PCR, and the clinical characteristics of the patients, as also the association between the two independent CTC detection methods, was estimated by using Fisher's Exact test. For multivariate analysis with the presence of CTCs as the dependent variable, logistic regression was performed. All tests were performed at the 0.05 level of significance.

Identification of EMT-associated biomarkers, which interfere with SCCHN cell migration and response to treatment

EGF stimulation of SCCHN cell lines

For EGF stimulation experiments, cells were seeded on day 0 and left undisturbed for 24 h. The culture medium was then replaced by serum-free medium supplemented with EGF (ProSpec-Tany TechnoGene Ltd, Rehovot, Israel) at 100 ng/ml.

Western blot

Expression of E-cadherin, EGFR and Snail1 in UD-SCC-4, UT-SCC-9, and FaDU, either untreated or treated with 100 ng/ml EGF, were assessed on protein level by standard Western blot analysis and also on mRNA level for Snail1 by real-time quantitative PCR analysis. The detailed description of the two methods and the reagents used is given in the corresponding publication **"Epithelial–mesenchymal-transition induced by EGFR activation interferes with cell migration and response to irradiation and cetuximab in head and neck cancer cells"** [Ref. (32)].

Immunofluorescence microscopy

UD-SCC-4, UT-SCC-9, and FaDU cells were seeded onto cover slips in 24-well plates (Costar, Corning Life Sciences, Amsterdam, The Netherlands) and cultured in the absence or presence of EGF. Cells were fixed with 2% formaldehyde at room temperature (RT),

washed with PBS and permeabilized on ice with 0.2% Triton X-100 in PBS/1% bovine serum albumin (BSA). After blocking with PBS/3% BSA, cells were incubated for 1 h at RT with an anti-E-cadherin antibody (clone 36, BD Biosciences) diluted at 1:100 in PBS/1% BSA/0.5% Tween 20. After washing, samples were incubated for 1 h with a secondary Alexa Fluor 488-labeled rabbit anti-mouse antibody (Invitrogen). Cell nuclei were then counterstained using 0.6 µg/ml DAPI (Serva, Heidelberg, Germany). Samples were washed with PBS and cover slips were transferred to microscope slides with mounting medium. Analysis was performed using a fluorescence microscope (Leica DM IL, Wetzlar, Germany).

Flow cytometry

For detection of surface EGFR UD-SCC-4, UT-SCC-9, and FaDU cells were trypsinized, washed with PBS and stained with an EGFR antibody [clone EGFR.1, phycoerythrin (PE)-labeled, BD Biosciences]. E-cadherin was detected by staining with an antibody raised against the C-terminal epitope of E-cadherin (clone C36, purified, BD Biosciences) after fixation and permeabilization of the analyzed cells. Cells were then washed with PBS and stained with a secondary rabbit anti-mouse antibody (Alexa Fluor 488-labeled, Invitrogen). All cells were stained in parallel with the appropriate isotype controls. Cells were analyzed by flow cytometry immediately after staining (FACSCanto II, BD Biosciences).

Migration and clonogenic survival assays

UD-SCC-4, UT-SCC-9, and FaDU cells were analyzed for their basal or EGF-induced migratory potential by means of the “scratch”, the “scatter” or the “Boyden chamber” assay. For estimating the sensitivity of these cell lines to irradiation, cetuximab or combined treatment, plating efficiency and survival fractions of treated cells were calculated on the basis of survival of untreated cells. A detailed description of these assays and the reagents used is given in Ref. (32).

Flow cytometric detection and molecular characterization of CTCs without tumor cell enrichment

Flow cytometric analysis of EGFR and phospho-EGFR expression in spiked SCCHN cells

Because of the high basal expression of EGFR in UT-SCC-23 cells, they were used for the optimization of our flow cytometric protocol for the analysis of EGFR/phospho-EGFR expression in CTCs. For induction of EGFR phosphorylation, UT-SCC-23 cells were incubated for 30 min with 100 ng/ml recombinant EGF (ProSpec-Tany TechnoGene Ltd). Cells were harvested by incubation with trypsin-EDTA. The staining with antibodies against surface EGFR (clone EGFR1, biotin-labeled, Abcam, Cambridge, UK) and intracellular phospho-EGFR [AlexaFluor647-labeled anti-phospho-EGFR (pY845) (clone 12A3) and (pY1173) (clone 9H2), BD Biosciences)] or the relevant isotype controls (BD Biosciences) was performed either solely on the UT-SCC-23 cells or after spiking them in 5 ml blood and processing the cell suspension according to the protocol for CTC detection without negative enrichment described below. For inhibiting potential phosphatase activity, the staining and permeabilization buffers were supplemented with sodium fluoride and sodium orthovanadate to a final concentration of 1 mM. The biotin-labeled EGFR antibody was detected by Streptavidin-PE-Cy™7 (BD Biosciences) in a secondary staining step.

Flow cytometric detection of CTCs without negative enrichment

Blood samples were processed for erythrocyte lysis according to the protocol described above. The remaining cell suspension was split in two equal fractions, which were stained either with specific antibodies to EpCam [clone EBA-1, peridinin-chlorophyll protein (PerCP)-Cy™5.5-labeled], the above mentioned anti-EGFR antibody and CD45 [clone HI30, fluorescein isothiocyanate (FITC)-labeled] (BD Biosciences Europe, Heidelberg, Germany) or the relevant isotype control antibodies (BD Biosciences). Cells were then fixed and permeabilized with the Fix&Perm Kit reagents according to the manufacturer’s

instructions. During the permeabilization step, the cells were stained with anti-cytokeratins-7/-8 antibody [clone CAM 5.2, phycoerythrin (PE)-labeled (BD Biosciences)] and the above mentioned anti-phospho-EGFR antibodies or the relevant isotype controls (BD Biosciences). Samples were analyzed on BD FACSCanto II. In order to be able to detect CTCs as very rare events in nonenriched blood samples, an electronic threshold was applied: threshold levels of 10,000 for the signal of the forward scatter, 400 for the signal from EpCam-PerCP-Cy5.5 and 300 for the signal from cytokeratin-PE were used as discriminators. Thus, cellular debris and CD45+ blood cells with background fluorescence signal below these threshold values were not detected by the flow cytometer. The PMT voltages used for recording of the fluorescence signals were as follows: FITC = 320, PE = 320, PerCP-Cy5.5 = 400, APC = 370 and PE-Cy7 = 380.

Patients and controls

Blood samples from 33 patients with locally advanced, inoperable SCCHN were included in this study. Of the 33 patients, 28 (84.8%) were male, 30 (90.9%) had stage IV disease and the most common tumor localizations were the hypopharynx (42.4%) and the oropharynx (39.4%). At the time point of the first blood sampling, none of the patients had started definitive tumor-specific treatment. In addition, blood samples from 20 healthy individuals were included in this study. The blood samples were processed according to the protocol for CTC detection without CD45+ cells enrichment described above. For the analysis of EGFR and phospho-EGFR expression in CTCs, the above-mentioned specific and isotype control antibodies were used. A blood sample was considered CTC+ when at least one EpCam+cytokeratin+CD45− cell was detected. The expression of EGFR/phospho-EGFR in CTCs was assessed by gating on this cell subset.

Monitoring of CTC frequency and their EGFR/phospho-EGFR expression during therapy

Patients

Blood samples (7.5 ml) were collected from 38 SCCHN patients with locally advanced, unresectable tumors (stage IVA/B), who participated in a phase II clinical trial comparing TPF induction (docetaxel, cisplatin, and 5-fluorouracil) followed by radiotherapy plus cetuximab vs. standard concurrent radiochemotherapy [HART plus cisplatin and 5-FU (PF-HART)]. 31 patients (82%) were male and the primary tumor localizations were predominantly at the oropharynx (47.5%) and hypopharynx (39.5%). Blood samples were analyzed for the presence of CTCs and their EGFR/phospho-EGFR expression according to the protocol for CTC detection without CD45+ cell enrichment described above at predefined time points – prior treatment, after completion of 3 cycles induction chemotherapy, at the end of treatment and at the first (6 weeks after the end of treatment) and the second follow-up (3 months after the end of treatment).

Statistical analysis

The comparison of the overall or treatment type-specific CTC frequency before and at the end of therapy was estimated by using Fisher's Exact test at the 0.05 level of significance.

Results

Evaluation of CTC correlation with clinical parameters

Detection of CTCs by flow cytometry after CD45 depletion: recovery, sensitivity and specificity

For optimization of the protocol for immunomagnetic enrichment and flow cytometric detection of EpCAM+cytokeratin+ CTCs, spiking experiments with the EpCAM+cytokeratin+ colorectal cell line SW620 were performed. Spiking from 5 to 500 SW620 cells into 5 ml healthy donor blood revealed a high linear detection rate (R^2= 0.99) with a mean recovery of 64%, ranging from 40% to 90%. Analysis of peripheral blood samples from 30 healthy donors revealed that the sensitivity of this method was 87%, since an EpCAM+cytokeratin+CD45- event was detected in 4 out of 30 samples and 3 of these 4 samples were also positive in the nested RT-PCR analysis.

Basal CTC numbers in peripheral blood from SCCHN patients with unresectable disease detected by flow cytometry after negative enrichment

Peripheral blood samples were collected before start of therapy from 42 SCCHN patients with unresectable disease and analyzed by the optimized protocol for flow cytometric detection of CTCs. Their presence was identified by detecting EpCAM+cytokeratin+CD45- events in 18 of 42 SCCHN cases (43%). The mean number of CTCs ± standard deviation (SD) detected in the CTC+ cases was 1.7 ± 0.9 cells per 3.75 ml blood, ranging from 1 to 4 cells.

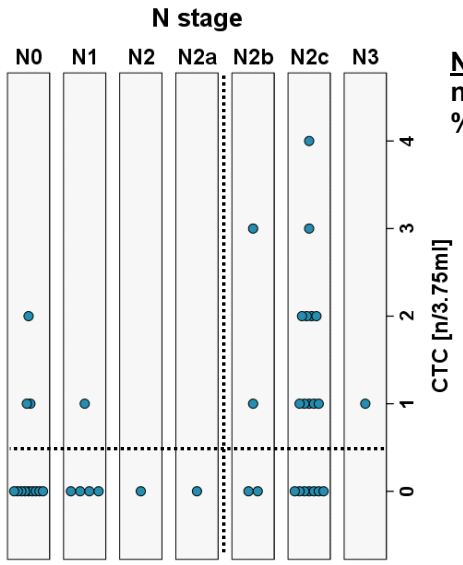
Correlation of CTCs with T stage and tumor volume

The patients with locally advanced SCCHN, in which the basal number of CTCs was assessed, were further stratified in two groups according to their T stage (T0-T3 *vs.* T4) and the association of CTCs presence or mean number with the tumor size was analyzed. This analysis revealed that the clinical T stage was associated neither with the absolute numbers of CTCs per 3.75 ml blood (p=.23), nor with the frequency of CTC+ cases (p>.999). We have further assessed if CTCs correlated with the tumor volume solely, since the T stage represents not only the size of the tumor, but also its invasiveness in adjacent areas. A tumor volume of 50 cm^3 was chosen as a cut-off for patient stratification. There was no significant association between the tumor volume and the numbers of CTCs (p=.23), nor their frequency (p=.35).

Correlation of CTCs with locoregional metastasis

A further point of interest in this study was whether CTCs correlated with the degree of affected lymph nodes. We have therefore grouped the cohort of 42 patients to subjects without lymph node metastasis or with a single lymph node affected (N0-N2a) and such with metastasis in multiple lymph nodes (N2b-N3), and compared the frequency of CTC+ cases and the number of CTCs between these groups. In comparison to the detected 4 CTC+ cases of 19 cases (21%) in the N0-N2a group, their frequency was significantly higher in the N2b-N3 group (p=.013, Figure 1A) with 14 CTC+ cases of 23 cases (61%). In the later group a greater mean number of CTCs was also observed (p=.007). Additionally, blood samples from 36 patients from the cohort were analyzed by nested RT-PCR for detecting EGFR transcripts, which not only confirmed CTCs presence in patient blood, but also its significant correlation with regional metastasis (p=.017, Figure 1B). A multivariate regression analysis was then performed by including the T stage, N stage and tumor volume. In this model, the association between the presence of CTCs, either detected by flow cytometry or nested RT-PCR, and the N stage remained significant (p=.014 and p=.024 accordingly).

A



N0-N2a vs N2b-N3:
nCTC/3.75ml: p=.007
%CTC+ cases: p=.013

B

P=.017	nested RT-PCR+	nested RT-PCR-	Total
N0-N2a	1	17	18
N2b-N3	8	10	18
Total	9	27	36

Figure 1. Correlation of circulating tumor cells (CTCs) with lymph node metastasis. (A) Patients were grouped according to their N stage. The detected numbers of CTCs in 3.75 ml blood in the different N-stage groups are presented. (B) Results from CTC analysis carried out by nested RT-PCR and their correlation with N stage.

Identification of biomarkers, which interfere with SCCHN cell migration and response to treatment

EGF treatment results in an EMT-like morphology of SCCHN cells

For the analysis of the difference in the expression of EMT-associated genes contributing to a different cell phenotype, three representative cellular morphologic types were selected: the epithelial cell-like UD-SCC-4 cell line, which forms dense colonies and monolayers; the mesenchymal cell-like UT-SCC-9 cells, which are spindle-shaped and grow in relatively loose networks and FaDu with a mixed epithelial-mesenchymal morphology, for a more detailed molecular characterization. It was first evaluated whether activation of EGFR could be responsible for the observed differences in morphology. Upon EGF treatment for 48 h, cells from all three cell lines reproducibly became elongated and spindle-shaped, resembling more the morphology of mesenchymal cells. Conversely, when EGFR activation was inhibited by addition of cetuximab, cells regained their epithelial cell-like morphology, formed tight cell-cell adhesions and grew in dense colonies and monolayers [Ref.(32)].

Autocrine and paracrine EGFR activation is associated with an EMT-like phenotype in SCCHN

After observing that modulation of EGFR activation influenced cellular morphology, it was further asked whether the mesenchymal-like morphology of unstimulated UT-SCC-9 cells resulted from constitutive EGFR activation. Expression analysis of EGFR using immunoblotting revealed that UD-SCC-4 had the highest levels of EGFR, UT-SCC-9 showed the lowest and FaDu intermediate expression levels [Ref. (32)]. Stimulation of cells with EGF for 30 minutes resulted in faster migration of the EGFR band in the immunoblot, indicating that a significant proportion of the total number of EGFR molecules were phosphorylated in response to EGF (Figure 2). Only the faster migrating bands were observed in untreated UT-SCC-9 cells, suggesting that the majority of the EGFR molecules were constitutively activated. Again, FaDu cells showed an intermediate pattern of EGFR activation with both slower and faster migrating bands being detected under nonstimulated conditions (Figure 2). Basal and EGF-triggered EGFR activation and its further internalization was confirmed by flow cytometry analysis of the membrane EGFR expression in the three cell lines. In line with an increased autocrine activity of EGFR in

UT-SCC-9 cells was also the observation that, when supernatants from the three cell lines were compared, the highest level of amphiregulin, one of the EGFR's major autocrine ligands, could be detected in UT-SCC-9 cell cultures [Ref. (32)].

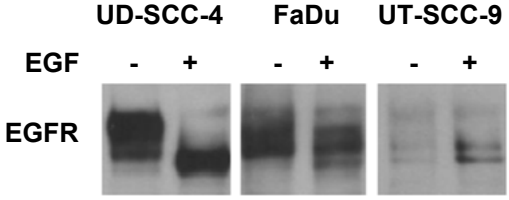


Figure 2. Autocrine and paracrine activation of EGFR is associated with an EMT-like phenotype in SCCHN. The expression level of EGFR in three representative SCCHN cell lines was assessed by immunoblotting.

EGF-induced EMT interferes with E-cadherin membrane localization, but fails to suppress E-cadherin expression

The expression of E-cadherin was further evaluated in the three cell lines, because downregulation of this protein by direct repression of its gene transcription has been reported to occur during EMT. In general there was no observable downregulation of E-cadherin 48 hs after EGF addition by immunoblotting or by flow cytometric analysis [Ref. (32)]. Localization studies using immunofluorescence microscopy, however, revealed a different staining pattern for E-cadherin in the cell lines [Ref. (32)]. While in unstimulated UD-SCC-4 cells E-cadherin was mainly concentrated at the plasma membrane in areas of cell-cell contacts, a strong signal for E-cadherin was also detected in perinuclear vesicular structures in UT-SCC-9 cells, suggesting that a significant part of the proteins had been internalized by endocytosis. The difference became even more apparent after EGF stimulation: while E-cadherin remained localized mainly at the sides of cell-cell junctions in UD-SCC-4 cells, the majority of E-cadherin was located in the perinuclear regions in UT-SCC-9 cells. Again, FaDu cells showed an intermediate pattern both under basal and stimulated conditions. Snail1 expression was further determined by immunoblotting as additional marker of EMT. In line with their distinct EMT-like morphology, basal Snail1 expression was detected in UT-SCC-9 and FaDu cells, but it was weakly expressed in UD-SCC-4 cells. In the latter cell line, Snail1 expression became clearly detectable only after stimulation of cells with EGF for 48 hs. Comparable results were obtained when Snail1 mRNA levels were quantified by real-time RT-PCR [Ref. (32)].

EGF-induced EMT-like phenotype is associated with an enhanced migratory potential

The migratory potential of the cell lines was next assessed by means of the scratch, the scatter and the Boyden chamber assays. In good concordance with EMT morphology and molecular profile, the results from all three independent assays revealed the lowest basal migratory potential for UD-SCC-4 cells and the highest one for UT-SCC-9 cells. Migration was increased when cells were stimulated with recombinant EGF. This effect was more rapid and stronger in UT-SCC-9 compared to FaDu and was almost not detectable in UD-SCC-4 cells [Ref. (32)].

EMT-like phenotype correlates with decreased sensitivity to irradiation and cetuximab monotherapy, which can be overcome by combined treatment

Finally, the difference in sensitivity to irradiation, cetuximab treatment or the combination of both among the SCCHN cell lines studied was analyzed. All three cell lines were sensitive to irradiation and only a slight, but significantly decreased, sensitivity was observed for UT-SCC-9 cells compared to FaDu and UD-SCC-4 cells [Ref. (32)]. In line with previous reports, those cell lines with an EMT-like phenotype were also less sensitive to cetuximab, although again, all three cell lines responded to growth inhibition by cetuximab and the differences in the IC50 values were rather small. In order to evaluate

whether the radiosensitizing effect of cetuximab is hampered by the EMT-like phenotype, the effect of irradiation and cetuximab alone on cell survival was compared with that of the combined treatment. Whereas in UD-SCC-4 cells the combination was as effective as cetuximab treatment alone, a synergistic effect of the combined treatment was observed in FaDu and UT-SCC-9 cells [Ref. (32)].

Flow cytometric detection and molecular characterization of CTCs without tumor cell enrichment

Flow cytometric detection of CTCs without CD45 depletion: recovery, sensitivity and specificity

The recovery of this protocol was determined by spiking 2 to 70 SW620 cells into 5 ml of blood. The flow cytometry analysis of spiked samples revealed a mean recovery of 85% (range 63-100%), which was higher than the recovery observed in the former protocol including negative enrichment by CD45+ cell depletion. The new protocol was also characterized by highly linear recovery rate (R^2=0.98). For the assessment of the specificity, blood samples from 20 healthy donors were analyzed. EpCam+cytokeratin+CD45- cells were detected in 2 of 20 blood samples (specificity: 90%), which was comparable to the specificity observed for the former protocol.

Basal CTCs numbers in PB from SCCHN patients with unresectable disease detected by flow cytometry without negative enrichment

Blood samples collected from 33 SCCHN patients with unresectable tumors were analyzed by the protocol for CTC detection without negative enrichment before start of treatment. CTCs were detected in 11 of 33 patients (33.3%), with a mean number of 1.5 ± 0.5 per 3.75 ml blood (range: 1–2 cells).

Assessment of EGFR expression and activation in SCCHN cells

After identifying EGFR activation as a sufficient event contributing to acquirement of EMT phenotype and enhanced cell mobility in SCCHN cell lines, which interfered with cell response to treatment, it was asked weather EGFR was activated in CTCs. To answer this question, it was first checked if EGFR phosphorylation can be detected in unstimulated and EGF-stimulated UT-SCC-23 cells with antibodies directed against the tyrosine residues of the EGFR at positions 845 (pY845) and 1173 (pY1173). No basal expression of phospho-EGFR was detected in UT-SCC-23 cells, but upon stimulation with EGF, phospho-EGFR became detectable in more than 95% of the cells. After spiking EGF-stimulated UT-SCC-23 cells at low numbers in blood samples from healthy donors, we were able to detect these EGFR+/phospho-EGFR+ epithelial tumor cells by the developed flow cytometry protocol for CTC detection without CD45+ cell depletion (Figure 3). This method was therefore used for detection of CTCs in pretreatment PB samples of 33 SCCHN patients having unresectable disease and the detected cells were analyzed for their EGFR/phospho-EGFR expression. EGFR was expressed at the surface of all detected CTCs whereas phospho-EGFR expression was detected in 36.4% of CTC+ cases.

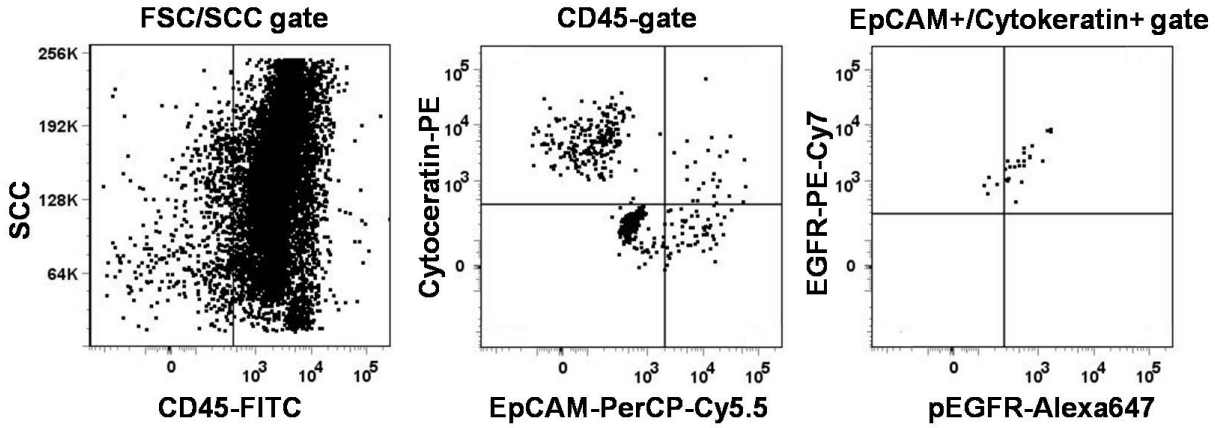


Figure 3. Analysis of the EGFR signaling pathway activation in CTCs. Spiked UT-SCC-23 cells were used as a model for CTCs expressing phospho-EGFR. Cells were stimulated with EGF and 100 cells were spiked in blood from a healthy donor. Epithelial tumor cells expressing EGFR and phospho-EGFR were detected among the CD45- cell population.

Monitoring of CTC frequency and their EGFR/phospho-EGFR expression during therapy

Blood samples were collected from 38 SCCHN patients with locally advanced, unresectable tumors who participated in a clinical study comparing TPF induction followed by radiotherapy plus cetuximab with standard concurrent radiochemotherapy. The samples were analyzed for the presence of CTCs and their EGFR/phospho-EGFR expression by flow cytometry without negative enrichment at predefined time points prior to, during and after the specific treatment and during follow-up visits (Figure 4). Overall, prior treatment CTCs were detected in the blood of 9 of 31 patients (29%). Administration of induction chemotherapy seemed not to have a major influence in the frequency of CTCs, since 5 of 14 patients (36%) were positive for CTCs after its completion. Interestingly, the frequency of CTC+ cases significantly increased after radiotherapy, independently of whether it was combined with cetuximab or cisplatin/5-FU, since at the end of treatment CTCs were detected in peripheral blood samples of 11 of 23 patients (48%). At the first and second follow up visit, 5 of 24 patients (21%) and 5 of 22 patients (23%), accordingly, still had detectable CTCs in their blood (Figure 4). We have further studied if the type of therapy applied had an influence on CTC detection rates. CTCs were detected in the blood of 26% and 33% of the patients before the start of TPF induction, followed by irradiation plus cetuximab, or concurrent chemoradiation, respectively (Figure 4). The increase in CTC frequency observed after irradiation was less visible in patients irradiated in combination with cetuximab, with 37.5% of all cases being CTC+ after treatment, in comparison to those treated in combination with cisplatin/5-FU, among which CTCs were detected in 71.4% of the patients. Although the statistical analysis revealed that the difference in the CTC frequency before and after therapy was not significant when all patients were included in the analysis (p=.25), nor when the two treatment regiments were analyzed independently (TPF+bioradiation: p=.72; chemoradiation: p=.16), this might have resulted from the small number of patients included in the study.

The modification of EGFR/phospho-EGFR expression in CTCs during the two therapy regimens, with and without administration of the anti-EGFR antibody cetuximab, was further studied (Figure 4). Before start of treatment, EGFR was expressed on the cell membrane in all CTC+ cases, which did not change in the course of treatment. Overall, the frequency of CTCs expressing phospho-EGFR among the CTC-positive SCCHN patients before treatment was 55%. When the influence of the type of therapy applied on EGFR phosphorylation was then analyzed, there was an observable increase in the

percentage of CTCs expressing phospho-EGFR in patients who underwent TPF induction therapy, which was further reduced by the administration of radiotherapy in combination with cetuximab. The percentage of phospho-EGFR/CTC+ cases detected before treatment remained almost unchanged in patients treated with concurrent radiochemotherapy (Figure 4).

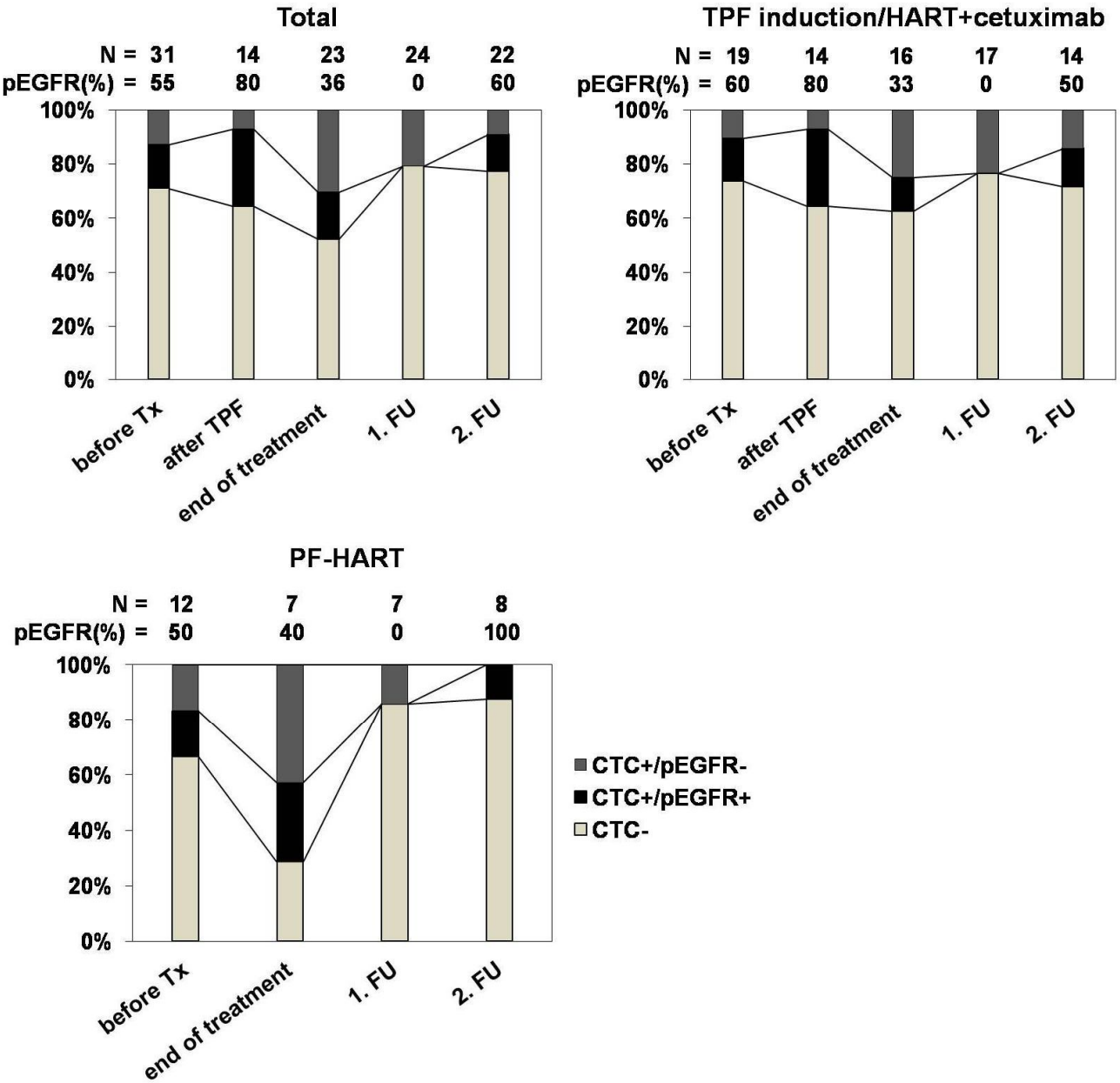


Figure 4. CTC presence and EGFR/phospho-EGFR expression in SCCHN patients undergoing TPF induction followed by irradiation (HART) plus cetuximab or concurrent chemoradiotherapy (PF-HART). Blood samples were collected from SCCHN patients at the identified time points and were analyzed by flow cytometry without preenrichment of the samples. The total number of analyzed samples (N) and the percentage of phospho-EGFR+ cases of the CTCs+ samples are given at the top of the graph.

Discussion and future aspects

When blood samples from SCCHN patients with locally advanced, inoperable disease were analyzed by both flow cytometry methods described in this study, CTCs were successfully detected at similar frequencies and low numbers. High numbers of CTCs have been reported mainly in cancer patients with distant metastatic disease (6,7,11,33,34) and the general occurrence of a low number of CTCs in the blood of locally advanced SCCHN patients is therefore expected, since very few cases present with clinically detectable distant metastases at the time of diagnosis (35). Moreover, the same low numbers of CTCs observed in our study have been recently detected in advanced SCCHN by using the CellSearch system, which is the only F.D.A. approved platform for CTC monitoring (36). Thus, after detecting CTCs in negatively enriched peripheral blood samples of 42 patients with advanced SCCHN by flow cytometry, it was asked whether their presence correlates with clinical parameters such as tumor size or nodal spread, since CTCs may either result from tumor development or represent the reservoir for metastasis formation. The observed lack of correlation between CTCs and the T stage or tumor volume and their significant correlation with the number of affected lymph nodes implies that the spread of the detected tumor cells in the blood of head and neck cancer patients is not a result of their passive detachment from growing primary tumors, but of an active process of tumor cell transformation and dissemination to the lymph nodes and distant organs via the circulation system, which has been suggested as the main route for metastasis of head and neck cancer (37-39). Indeed, the number of lymph nodes involved is an established prognostic factor for the development of distant metastasis in SCCHN (40-44). Therefore, the presence of CTCs in patient blood might serve as an additional marker predicting the metastasis-free survival of SCCHN patients. Additionally, in this study CTCs have been also detected, though rarely, in patients without clinically detectable lymph node metastasis, which suggests that CTCs may also represent a novel diagnostic tool for the detection of early metastasis.

Furthermore, CTCs represent an easily accessible tumor material on which the expression of molecules related to the metastatic nature of the tumor or its response to therapy can be studied. In order to identify such markers in SCCHN, the EGFR activation-induced EMT phenotype, migratory potential and response to treatment of three cell lines, representative for different EMT morphologic types, has been characterized. The basal EMT-like phenotype in SCCHN cells was associated with autocrine activation of EGFR and was also characterized by high levels of Snail1 and increased migratory potential. However, there was no observable transcriptional repression of E-cadherin by Snail1 reported to occur during EMT in other cell models (45), meaning that the disruption of the tight cell-cell junctions via downregulation of E-cadherin expression is not solely required for the acquisition of a motile phenotype. The disruption of cell-cell adhesion in the SCCHN *in vitro* cell lines analyzed in this study was most probably a result from the observed internalization of E-cadherin from the cell membrane to the perinuclear regions after EGFR activation (46), which contributed to the enhanced mobility of the tumor cells. E-cadherin translocation in SCCHN cell lines with an EMT phenotype might also stimulate EGFR-mediated cell growth, since tight cell-cell contacts, formed by homophilically bound E-cadherin, normally inhibit EGFR phosphorylation by EGF and its downstream signaling, thus inhibiting cell proliferation (47). Moreover, the EMT phenotype of the analyzed SCCHN cells was negatively associated with their response to irradiation and the anti-EGFR antibody cetuximab (32). Ionizing radiation can induce EGFR phosphorylation, which leads to the transport of the receptor to the cell nucleus, where it activates the repair of radiation-induced double DNA strand breaks (48,49). Thus reduction of homophilically bound E-cadherin at SCCHN cell surface can weaken its inhibitory effect on irradiation-induced EGFR phosphorylation and thus contribute to the activation of cell survival mechanisms and the acquisition of cell radioresistance. Additionally, the expression of

Snail1 during EMT observed in the SCCHN cell lines analyzed in this study might also mediate their treatment resistance through the transcriptional repression of phosphatase and tensin homolog (PTEN) (50), which normally inhibits the PI3K/AKT pathway. Thus, PTEN downregulation enhances the activation of this cell outgrowth and survival pathway, which is strongly associated with the resistance of SCCHN cells to radiotherapy (51) and cetuximab (52).

Based on the observed relationship of EGFR expression and activation to cell migration and therapy resistance in SCCHN, these markers were chosen for the further molecular analysis of CTCs. Therefore, a simple multicolor flow cytometry protocol for detection and molecular characterization of CTCs without negative enrichment was developed and blood samples from 33 SCCHN patients with unresectable disease were first analyzed before treatment initiation for the presence of CTCs and their expression of EGFR/phospho-EGFR. EGFR was detected in CTCs from all CTC+ SCCHN cases, which suggest that EGFR expression, reported in the majority of primary SCCHN (53-55), remains stable in the metastasized cells during their circulation and they can be successfully targeted by anti-EGFR drugs. However, basal phospho-EGFR expression was found in 36% of the CTC+ cases which was significantly higher than the frequency reported for primary SCCHN tissue (56). The only study in which the expression of phospho-EGFR has been analyzed in CTCs revealed its increased expression in patients with metastatic compared to early breast cancer (57). Therefore, the detection of CTC+ cases expressing phospho-EGFR might identify SCCHN patients with more aggressive disease. The prognostic value of phospho-EGFR expression in CTCs and its potential to identify patients who might specifically benefit from the addition of EGFR-targeting drugs to the treatment regimen need to be determined in future clinical trials.

This analysis was initiated by monitoring the change in CTCs presence and their EGFR/phospho-EGFR expression in the blood of 38 patients with advanced-stage SCCHN during the course of clinical trial comparing induction chemotherapy/ radiotherapy+cetuximab with standard radio-/chemotherapy treatment, in order to understand whether changes in CTCs presence and phenotype reflect treatment efficacy. The increase in the number of CTC-positive cases observed after radiotherapy could be due to either a possible increase in the number of detected tumor cells due to detachment of dead cells from the primary tumor after a more aggressive chemotherapy (58) or the ability of irradiation to trigger the EMT-related migration of SCCHN cells via activation of the EGFR signaling pathway (59). The first option is less possible, since the ability of CTCs to express proteins, detected during their flow cytometry analysis, suggests a preserved viability of these cells. This would be further confirmed by additionally assessing the viability of the detected CTCs by including a permeabilization-compatible viability dye in the flow cytometry protocol. The second hypothesis is supported by a study by Biswas et al., in which administration of ionizing radiation in transgenic mouse model of metastatic breast cancer caused an increase in number of CTCs and lung metastases, which also correlated with an increase in plasma TGF-β1 (60) that has also been shown to play a role in EMT and metastasis (61). Therefore, the administration of cetuximab to the treatment might block radiation-induced metastasis via inhibiting EGFR signaling, which has been already shown in a glioma tumor mouse model (62). Additionally, we have also observed a synergistic effect between the two treatments in SCCHN cells with a distinct EMT phenotype. The direct comparison of the frequency of CTC+ cases between two clinical study arms performed in this study has actually confirmed that the addition of cetuximab to radiation therapy of SCCHN patients is more effective in reducing the number of CTC+ cases than concurrent chemoradiation.

In addition to monitoring the changes in CTC frequency during treatment, the detected cells were also characterized for changes in their EGFR expression and activation status. EGFR signal was detectable on all CTCs before initiation of treatment and the signal

remained even when the anti-EGFR antibody drug was administrated in the treatment schedule. This can be explained by the fact that even though internalization of membrane EGFR after binding of cetuximab has been previously observed, a portion of the protein remains still at the surface (63,64). The remaining membrane fraction of EGFR is most probably detected by the anti-EGFR antibody used in our study which can even bind cetuximab-bound EGFR (64). The observation of the EGFR phosphorylation status in CTCs during therapy revealed that in the group of patients receiving only radio-/chemotherapy a significant proportion of the CTCs still expressed phospho-EGFR after treatment. This can be explained by the above mentioned ability of irradiation to activate EGFR autophosphorylation in SCCHN cell lines (49,65). Such irradiation-induced EGFR phosphorylation could be abolished by addition of cetuximab. This hypothesis was confirmed in the present study by the strong reduction observed in the frequency of phospho-EGFR signal among CTC-positive patients who underwent radio-/cetuximab therapy. However, EGFR phosphorylation was still detectable in a few cases. This may be due to an acquired resistance to cetuximab or to an incomplete inhibition of EGFR phosphorylation by the antibody (63,66). Additionally, binding of cetuximab to its target has been shown to paradoxically induce basal EGFR phosphorylation in lung and head and neck cancer cell lines, but still retain its ability to inhibit the activation of EGFR downstream signaling molecules such as Akt and Erk1/2 (67,68).

Thus, whether CTCs predict a more aggressive disease and worse disease outcome will be in future determined after further obtaining a larger number and a longer follow-up of SCCHN patients, allowing the correlation of basal CTC levels with their metastasis-free or overall survival. Additionally, the effect of treatment on CTCs survival and dissemination will be studied by assessing the viability of the detected cells and by including additional markers in their phenotypic analysis, such as Snail or Akt.

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Contribution declaration

The contribution of Ms. Tsvetana Hristozova to the submitted publications was as follows:

1. **Hristozova T**, Konschak R, Stromberger C, Fusi A, Liu Z, Weichert W, et al. The presence of circulating tumor cells (CTCs) correlates with lymph node metastasis in nonresectable squamous cell carcinoma of the head and neck region (SCCHN). Ann Oncol 2011;22:1878-85.

Share: 50%

Contribution in detail: Handling and culture of in vitro tumor cells lines, spiking experiments, optimization of the protocol for immunomagnetic enrichment of the tumor cells, optimization of the flow cytometry staining protocol, processing of patients blood samples, flow cytometry analysis, data analysis, statistical analysis

2. Holz C, Niehr F, Boyko M, **Hristozova T**, Distel L, Budach V, et al. Epithelial mesenchymal-transition induced by EGFR activation interferes with cell migration and response to irradiation and cetuximab in head and neck cancer cells. Radiother Oncol 2011;101:158-64.

Share: 10%

Contribution in detail: Handling of *in vitro* tumor cells lines, ligand stimulation of EGFR in *in vitro* tumor cell lines, flow cytometry analysis, immunocytochemistry, data analysis and representation, manuscript revision

3. **Hristozova T**, Konschak R., Budach V. and Tinhofer I. A simple multicolor flow cytometry protocol for detection and molecular characterization of circulating tumor cells in epithelial cancers. Cytometry A 2012; 81(6):489-95.

Share: 50%

Contribution in detail: Handling and culture of *in vitro* tumor cells lines, spiking experiments, development of circulating tumor cells immunostaining and flow cytometry detection protocol without preenrichment of the blood samples, handling and analysis of patient blood samples, data analysis and representation, manuscript writing and revision

4. Tinhofer I, **Hristozova T**, Stromberger C, Keilholz U, Budach V. Monitoring of circulating tumor cells and their expression of EGFR/phospho-EGFR during combined radiotherapy regimens in locally advanced squamous cell carcinoma of the head and neck. Int J Radiat Oncol Biol Phys 2012; 83(5):e685-90.

Share: 30%

Contribution in detail: Detection and phenotypic analysis of circulating tumor cells by flow cytometry without preenrichment of the blood samples in the frame of a clinical study, data analysis and representation, manuscript revision

…….
Tsvetana Hristozova
(PhD candidate)

…….
PD Dr. I. Tinhofer-Keilholz
(Thesis supervisor)

Selected publications

Publication 1

Hristozova T, Konschak R, Stromberger C, Fusi A, Liu Z, Weichert W, Stenzinger A, Budach V, Keilholz U, Tinhofer I.

The presence of circulating tumor cells (CTCs) correlates with lymph node metastasis in nonresectable squamous cell carcinoma of the head and neck region (SCCHN).

Annals of Oncology 2011; 22:1878-85; Journal Impact factor: 6.452

DOI: 10.1093/annonc/mdr130

Abstract

Background: The mechanisms regulating tumor cell dissemination in locally advanced squamous cell carcinoma of the head and neck region (SCCHN) are largely unresolved. We assessed the frequency of circulating tumor cells (CTCs), their association with clinicopathologic parameters and their kinetics during radiochemotherapy.

Patients and methods: Peripheral blood samples from 42 patients with locally advanced SCCHN were included. CTCs were detected using flow cytometric analysis of CD45-epithelial cell adhesion molecule+cytokeratin+ cells and results were validated by nested RT-PCR analysis of circulating epidermal growth factor receptor transcripts. The association between the presence of CTCs and T stage, tumor volume, N stage and human papillomavirus status was evaluated. The influence of radiochemotherapy on CTC numbers was determined.

Results: CTCs were detected in 18 of 42 SCCHN patients (43%), with a mean ± standard deviation of 1.7 ± 0.9 CTCs per 3.75 ml blood. We observed no significant correlation between the presence of CTCs and T stage or tumor volume. However, a nodal stage of N2b or higher was associated with higher frequency of CTCs. Though concurrent radiochemotherapy reduced their frequency, CTCs persisted during treatment in 20% of cases.

Conclusions: Detection of CTCs correlates with regional metastasis in inoperable SCCHN. Further follow-up is needed to evaluate the prognostic significance of CTC detection, in addition to clinical staging of lymph nodes, for regional or distant recurrence.

Publication 2

Holz C, Niehr F, Boyko M, **Hristozova T**, Distel L, Budach V, Tinhofer I.

Epithelial-mesenchymal-transition induced by EGFR activation interferes with cell migration and response to irradiation and cetuximab in head and neck cancer cells.

Radiotherapy & Oncology 2011; 101:158-64; Journal Impact factor: 4.337

DOI: 10.1016/j.radonc.2011.05.042

Abstract

Background and purpose: The role of epithelial-mesenchymal transition (EMT) in the poor outcome of EGFR-overexpressing SCCHN was evaluated.

Material and methods: SCCHN cell lines were characterized for their cell morphology and expression of EGFR and the EMT-associated factors E-cadherin, vimentin and Snail1. The migratory potential of cells was assessed in motility assays. Response to irradiation and cetuximab was determined using clonogenic survival assays.

Results: High basal expression of E-cadherin but low to absent vimentin expression could be observed in all SCCHN cell lines. Although E-cadherin expression levels did not change after treatment with EGF we observed a significant change in cell morphology resembling EMT. SCCHN cells with high basal levels of Snail1 resulting from constitutive EGFR activation were characterized by mesenchymal-like morphology, elevated migratory potential, reduced sensitivity to irradiation and cetuximab but increased sensitivity to the combined treatment.

Conclusions: Autocrine activation of EGFR leading to EMT is associated with a metastatic phenotype and reduced sensitivity of SCCHN cells to single-modality treatment with cetuximab or irradiation. The potential of Snail1 as biomarker for selection of patients who will mostly benefit from a combination of cetuximab and radiotherapy has to be evaluated in future clinical studies.

Publication 3

Hristozova T, Konschak R, Budach V and Tinhofer I.

A simple multicolor flow cytometry protocol for detection and molecular characterization of circulating tumor cells in epithelial cancers.

Cytometry Part A 2012; 81(6):489-95; Journal Impact factor: 3.753

DOI: 10.1002/cyto.a.22041

Abstract

Circulating tumor cells (CTCs) might not only serve as prognostic marker but could also be useful for monitoring treatment efficacy. A multicolor flow cytometry protocol for their detection and molecular characterization in peripheral blood was developed which consisted of erythrocyte lysis followed by staining of cells with fluorochrome-labeled antibodies against CD45 and the epithelial markers EpCam and cytokeratin 7/8. For reducing the number of events acquired by flow cytometry, an electronic threshold for the fluorescent signals from the epithelial markers was applied. After establishment of the protocol by using spiking experiments, its suitability to determine the absolute number of CTCs as well as their expression of epidermal growth factor receptor (EGFR) and its phosphorylated form (phospho-EGFR) in blood samples from patients with squamous cell carcinoma of the head and neck (SCCHN) was validated.

Spiking experiments demonstrated an excellent recovery (mean 85%) and a linear performance (R^2=.98) of the protocol. Sensitivity and specificity were comparable to our former protocol using immunomagnetic CTC pre-enrichment. The analysis of 33 SCCHN patient samples revealed the presence of CTCs in 33.3% of cases with a mean ± SD of 1.5 ± 0.5 CTCs per 3.75 ml blood. EGFR was expressed in 100% and phospho-EGFR in 36.4% of the CTC+ cases.

We have established a simple and sensitive multicolor flow cytometry protocol for detection of CTCs in patients with epithelial cancers including SCCHN which will allow their detailed molecular characterization.

Publication 4

Tinhofer I, **Hristozova T**, Stromberger C, Keilholz U, Budach V.

Monitoring of circulating tumor cells and their expression of EGFR/phospho-EGFR during combined radiotherapy regimens in locally advanced squamous cell carcinoma of the head and neck.

International Journal of Radiation Oncology * Biology * Physics 2012; 83(5):e685-90; Journal Impact factor: 4.503

DOI: 10.1016/j.ijrobp.2012.02.009

Abstract

Purpose: The numbers of circulating tumor cells (CTCs) and their expression / activation of EGFR during the course of combined chemo- or bioradiotherapy regimens as potential biomarkers of treatment efficacy in SCCHN were determined.

Methods and Materials: Peripheral blood samples from SCCHN patients with locally advanced stage IV A/B disease who were treated with concurrent radiochemotherapy or induction chemotherapy followed by bioradiation with cetuximab were included in this study. Using flow cytometry, the absolute number of CTCs per defined blood volume as well as their expression of EGFR and its phosphorylated form (pEGFR) during the course of treatment were assessed.

Results: Before treatment, we detected ≥ 1 CTC per 3.75 ml blood in 9 of 31 patients (29%). Basal expression of EGFR was detected in 100% and pEGFR in 55% of the CTC+ cases. The frequency of CTC detection was not influenced by induction chemotherapy. However, the number of CTC+ samples significantly increased after radiotherapy. This radiation-induced increase in CTC numbers was less pronounced when radiotherapy was combined with cetuximab compared to its combination with cisplatin/5-fluorouracil. The former treatment regimen was also more effective in reducing pEGFR expression in CTCs.

Conclusions: Definitive radiotherapy regimens of locally advanced SCCHN can increase the number of CTCs and might thus contribute to a systemic spread of tumor cells. Further studies are needed to evaluate the predictive value of the radiation-induced increase in CTC numbers and the persistent activation of the EGFR signalling pathway in individual CTC+ cases.

Curriculum Vitae

My resume will not be published in the electronic version of my work for privacy reasons.

List of publications

Tinhofer I, **Hristozova T**, Stromberger C, Keilhoiz U, Budach V. Monitoring of Circulating Tumor Cells and Their Expression of EGFR/Phospho-EGFR During Combined Radiotherapy Regimens in Locally Advanced Squamous Cell Carcinoma of the Head and Neck. Int J Radiat Oncol Biol Phys 2012. [Epub ahead of print]

Hristozova T, Konschak R, Budach V, Tinhofer I. A simple multicolor flow cytometry protocol for detection and molecular characterization of circulating tumor cells in epithelial cancers. Cytometry A 2012. [Epub ahead of print]

Budt M, **Hristozova T**, Hille G, Berger K, Brune W. Construction of a lytically replicating Kaposi's sarcoma-associated herpesvirus. J Virol 2011;85:10415-20.

Holz C, Niehr F, Boyko M, **Hristozova T**, Distel L, Budach V, et al. Epithelial-mesenchymal-transition induced by EGFR activation interferes with cell migration and response to irradiation and cetuximab in head and neck cancer cells. Radiother Oncol 2011;101:158-64.

Hristozova T, Konschak R, Stromberger C, Fusi A, Liu Z, Weichert W, et al. The presence of circulating tumor cells (CTCs) correlates with lymph node metastasis in nonresectable squamous cell carcinoma of the head and neck region (SCCHN). Ann Oncol 2011;22:1878-85.

Oral presentations

Holz C, Niehr F, Boyko M, **Hristozova T**, Distel L, Budach V, Tinhofer I. Induction of epithelial-mesenchymal-transition by recombinant epidermal growth factor increases the metastatic potential of head and neck squamous carcinoma cells (SCCHN) and negatively interferes with their response to irradiation and EGFR targeting. 17th Annual meeting of German Society of Radiation Oncology, Wiesbaden, Germany, 02.06-05.06.2011

Hristozova T, Konschak R, Fusi A, Stromberger C, Budach V, Keilholz U, Tinhofer I. Characterization of EGFR expression in circulating tumor cells in SCCHN. Annual meeting of German, Austrian and Swiss Oncology and Haematology Associations, Berlin, Germany, 01.10 – 06.10.2010

Hristozova T, Konschak R, Fusi A, Stromberger C, Budach V, Keilholz U, Tinhofer I. Characterization of circulating tumor cells in squamous cell carcinoma of the head and neck region. 19th Symposium “Experimental Radiotherapy and Clinical Radiobiology”, Dresden, Germany, 04.03 – 06.03.2010

Selbständigkeitserklärung

Ich, Tsvetana Hristozova, erkläre, dass ich die vorgelegte Dissertation mit dem Thema:

“Characterization of circulating tumor cells in locally advanced squamous cell carcinoma of the head and neck”

selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.

Berlin, den 24.09.2012 Tsvetana Hristozova

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