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Full paper. Title of the original work:

Heterogeneity of epithelial and stromal cells of head and neck squamous cell carcinomas in *ex-vivo* chemoresponse

Running title:

Heterogeneity of HNSCC in *ex-vivo* chemoresponse

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Abstract (191 words)

Background: Valid prediction of effectiveness of chemotherapeutic agents in individual head and neck squamous cell carcinoma (HNSCC) is desirable and might be achieved using *ex-vivo* assays.

Methods: Three biopsies from each of 15 HNSCC were taken, minced, and digested by collagenase. The digested HNSCC was added to serial dilutions of either cisplatin (CIS) or docetaxel (DTX) which were prepared under flavin-protecting conditions in ECM-coated microtiterplates. After 72-h incubation, cultures were methanol-fixed and Giemsa-stained. The cut-off concentration (COC; concentration completely suppressing colony formation) for epithelial cells (EC) and stromal cells (SC) was evaluated.

Results: 12/15 HNSCC (80%) were evaluable. Despite significant correlation of COC of CIS in respect of colony formation of EC or SC, no significant differences in response of individual HNSCC specimens were found in the *t*-test for paired samples ($p>0.16$). The same applies to DTX. However, EC and SC showed heterogeneity in chemoresponses leading to COC variability of more than one titration step in 44.1% (CIS) and 20% of HNSCC (DTX). No significant correlation between the COC of both cell populations was found in HNSCC specimens.

Conclusions: The *ex-vivo* chemoresponse of EC and SC of HNSCC must be analyzed separately.

Key words

Heterogeneity

HNSCC

ex-vivo chemoresponse

epithelial cells

stromal cells

Introduction

During the last five decades many *in-vitro* chemoresponse assays were developed with the aim to achieve information about a tumor's sensibility or resistance to cytostatic drugs [01-03]. Especially in the 1980th and 1990th predictive tests were quite popular and subject of many studies [04-08]. But due to severe limitations of these test protocols [09-11], and corresponding increased skepticism, chemoresponse assays were never included into clinical routine.

Over the same time period chemotherapy in head and neck oncology has changed from an only palliative treatment to an important component in multimodal therapies. For example, in larynx organ preservation protocols, induction chemotherapy followed by irradiation has been proven to be sufficient with moderate late toxicity outcomes in nearly every second patient with head and neck squamous cell carcinoma (HNSCC) [12]. But chemotherapy (CT) and chemoradiation are not always curative since the responses to treatment of individual tumors differ widely. Moreover, the necessary doses which are required to affect the individual tumor's growth are not known. Applying inadequate doses of chemotherapeutics probably also induces further increase of chemo-resistance in HNSCC to the applied agents and possibly additionally also to other potential therapeutics [13-15]. There is a high risk to end up with inefficient but nevertheless toxic treatment of HNSCC patients.

Up to the last ten years, there was only limited need for predictive assays of HNSCC because of the small number of approved cytostatic agents and the

limited number of guideline-conform first and second line treatment protocols in use. But today – and in particular in the contradictory context of increasing number of new emerging cytostatic compounds and biologicals allowing for chemotherapy or radiochemotherapy expanded by targeted therapy but also the possibility to choose the false treatment modality – there is increasing pressure to select correctly the most appropriate pharmaceuticals and to apply individualized treatments adapted to the requirements of the particular HNSCC. This only can be resolved on basis of knowledge about the individual tumor's reactivity to the eligible compounds in the context of maximum acceptable dosage. Thus the need for an *ex-vivo* chemoresponse assay in head and neck oncology to predict the success of individual types of multimodal therapy protocols has increased. However, conventional *in-vitro* testing was judged to be useless as predictive assay [10, 11]. Indeed, more efficient solutions to overcome critical limitations of conventional *in-vitro* assays by utilizing flavin-protecting conditions [15] to eliminate flavin-mediated photochemical artifacts [16] are available. Advantageously, they are quality-controlled, and allow also for earlier availability (4 days) of data respective to dosages required to achieve the intended response of the individual HNSCC [17, 18].

To use such an predictive assay appears to be even more important since only a small amount of markers for chemoresponse of HNSCC is known up to now, partially with contrary interpretations of their meaning [19-22], and since all of the so far known markers do not allow statements about the individual sensibility or resistance of a given tumor. Furthermore, cell line based

chemosensitivity testing in highly selected tumors do not reflect the needs of average tumor behavior in individual patients.

We already used this predictive short-time *ex-vivo* colony-forming assay to confirm that the chemoresponse of epithelial cells within a biopsy in principal is representative for a given HNSCC [17]. But within that study the *ex-vivo* chemoresponse of stromal cells was not analyzed. Moreover, it might be mistakenly concluded from the data shown in [17] that any biopsy of a tumour allows for sufficient statements about the chemoresponse of all cell types present within the tumour. Thus the aim of this consecutive study was to analyze potential differences in the chemoresponse of epithelial and stromal cells in individual HNSCC. Therefore three separate biopsies of a HNSCC (“fragments”) were taken from spatially distant regions. Subsequently the chemoresponse of epithelial and stromal cells was obtained using our *ex-vivo* colony-forming assay [18] and analyzed regarding differences in the chemoresponse of epithelial and stromal cells and as well representativeness of a single specimen for the other tissue samples and thus for the whole HNSCC.

Materials and Methods

Patients and HNSCC specimens

Included in this study were 15 patients with histologically-confirmed primary HNSCC. The stages following criteria of the American Joint Committee on Cancer (AJCC [23]) were as follows: 8.3% stadium I, 16.7% stadium II, 16.7% stadium III, and 58.3% stadium IV (table 1). After obtaining patient’s informed consent, three biopsies (>60 mg) from spatially distant tumor areas were taken

during surgical tumor resection under general anaesthesia. The tissue samples were placed in tubes containing tumor medium (TM) and immediately transferred to the laboratory. There they were weighed and processed under flavin-protecting conditions (see below).

Materials

TM was used as cell culture medium for chemoresponse testing of the HNSCC samples as well as of the KB cells for quality control. TM consisted of a phenol red-and flavin-free RPMI 1640 (Biochrom[®], Berlin, Germany) supplemented by 10% (v/v) fetal calf serum (FCS; Biochrom), amikacin, nystatin, penicillin, and streptomycin (all from Sigma, Munich, Germany). After addition of FCS the TM contained 20 nM of riboflavin. Consequently, all following experimental steps were carried out utilizing flavin-protecting conditions (avoidance of adverse flavin-mediated photo-induced reactions by exclusive illumination using Philips SOX-E sodium-discharge lamps with monochromatic excitation at the wavelength of $\lambda = 589$ nm). Cisplatin was purchased from Sigma. Docetaxel (Taxotere[®]) was purchased as pharmaceutical preparation from Sanofi-Aventis Deutschland GmbH (Berlin, Germany).

KB cell chemoresponse assay

For quality control purposes and examination of reproducible test conditions along to each HNSCC colony formation assay, a KB cell chemoresponse assay

was carried out simultaneously as previously described [17]. Briefly, KB cells from stock cultures adapted to RPMI 1640 free of phenol red and containing only 20 nM of riboflavin were harvested and adjusted to give a final number of 10^4 KB-cells/well. The KB-cell suspension was added to wells of microtiterplates containing dilutions of either cisplatin or docetaxel derived from those dilutions created for the *ex-vivo* test of the individual HNSCC. KB cells were harvested after 72 hours of incubation (36.5°C, 3.5% CO₂, humidified air) and counted using a semi-automated cell counter (Casy TT[®], Schärfe System, Reutlingen, Germany). The inhibitory concentrations (IC) of cytostatic drugs leading to diminished proliferation or even cytotoxicity and causing a drop down in cell counts to 50% of cell numbers counted in control wells receiving only medium (IC₅₀) were calculated. These IC₅₀ values were compared to the previously published IC₅₀ values for KB cells [24-25].

HNSCC colony formation assay

During tumor resection of 15 HNSCC biopsies within a mass range from 34 mg to 676 mg were obtained. The tissue samples of each HNSCC were handled separately. Each sample was minced into pieces of about 1 mm³, transferred into pre-warmed TM and disintegrated using collagenase type IV (230 U/ml; Sigma). After 16-h incubation the digests were washed by centrifugation. The pellet of disintegrated HNSCC was resuspended and pipetted into wells of ECM-coated microtiter plates (Paesel & Lorey, Hanau, Germany) containing serial dilutions of cisplatin or docetaxel. In consequence of the sometimes limited sample size, and despite the aim of starting the chemoresponse test by

adding 2 mg of collagenase digest, only 1.91 ± 0.13 mg (mean \pm SEM) of HNSCC digests were seeded per well of the microtiter plate. The applied concentrations of the chemotherapeutics were chosen based on the data specifying the tolerable plasma level (TPL) in man (cisplatin: $6.67 \mu\text{M}$ [26], docetaxel: $0.55 \mu\text{M}$ [27]). After 72 h at 36.5°C and 3.5% CO_2 in humidified air, supernatants were discarded and the wells gently washed twice using phosphate-buffered saline. Adherent cells and cell colonies were fixed with 90% (v/v) methanol and air-dried. Giemsa staining allowing for differentiation of epithelial and stromal cell colonies was performed as described [17].

Cut-off analyses and statistical investigations

Colonies were counted independently by two well-trained investigators using a Zeiss Axiovert 200M inverted microscope (Zeiss, Jena, Germany). Afterwards the results were compared, discussed and deviant findings reanalyzed together to achieve concordance in interpretation of microscopic observations and to accomplish decision-making by consensus.

A colony was defined as cell group consisting of at least eight cells of the regarding cell type. Endothelial colonies and those of fibrocytes were summarized as stromal colonies. Their numbers were compared with those of epithelial colonies. Cut-off analyses only were performed if sufficient colony formation (*i.e.* at least two epithelial and two stromal colonies of \geq eight cells each) was detected in all control wells without any chemotherapeutics.

Cut-off values for epithelial as well as stromal cell colonies of the individual HNSCC fragments were compared in the *t*-test for paired samples regarding

correlation and significance of differences using SPSS Statistics 15.0 for Windows version 15.0.1 (SPSS Inc., Chicago, Illinois). A p value below 0.05 was regarded to be significant.

Results

Types of ex-vivo formed colonies derived from HNSCC specimen

After Giemsa staining differentiation of epithelial and stromal colonies was carried out in each well of the microtiter plates by microscopic examination as described before [17]. In general, epithelial cells are represented by big cuboid cells with wide cytoplasm margin and a big round cell core. Fibroblasts, fibrocytes and endothelial cells were summarized as stromal cells. Fibrocytes and fibroblasts grew fusiform. They had a small cytoplasm margin and small oval cell cores. The fibroblast colonies showed fishbone-like growth patterns. The endothelial cells, in contrast, grew triangle like and had a wider cytoplasm margin but commonly also a bigger round cell core than the fibroblasts. Examples of the different colony types consisting of epithelial and stromal cells are shown in figure 1.

Evaluability of chemoresponse tests

Only a colony formation assay was regarded as evaluable, if at least two of the three fragments showed sufficient colony formation of epithelial as well as stromal cells in control wells, and, in addition, the KB cell chemoresponse assays confirmed the correct efficiency of the cytostatic drugs in determination of the regarding IC_{50} value ($\pm 30\%$). Twelve out of 15 colony formation assays with cisplatin as well as docetaxel met these inclusion criteria for cut-off

determination. This corresponds with a success rate of 80%. In some tumors only two samples were evaluable. Three assays showed no colony formation in all of the three fragment samples but this was not caused by technical limitations or contaminations. Neither of the tests had to be excluded because of bacterial or fungal contamination. All 14 concomitant KB cell experiments confirmed the predetermined IC_{50} values of 200 nM cisplatin [24] ($220 \text{ nM} \pm 8 \text{ nM}$ corresponding to $110 \pm 4\%$), and 280 pM docetaxel [25] ($280 \pm 8.1 \text{ pM}$ corresponding to $100 \pm 2.9\%$). These data show that all experiments were performed under reproducible conditions and therefore allow for reliable quantification of the effective chemotherapeutic dosage in the colony formation assay.

Cut-off determination respective to formation of epithelial cell colonies

Since cut-off determination was carried out separately for epithelial and stromal cell colonies for both cytostatic drugs, the outcome of cut-off determination is shown separately for these major types in table 2.

Following cisplatin treatment, the range of cut-off concentrations for colony formation of epithelial cells varied essentially between the different tumors. For individual HNSCC, cut-off concentration of 3.3 up to $>50 \mu\text{M}$ was observed. In contrast, the cut-off for the three fragments of a given tumor was reached at almost identical concentration. In eight of the twelve tumors the cut off was identical in all fragments. Regarding the remaining four HNSCC, the cut-off variation between the fragments was only one titration step. Cut off was reached in all assays below the highest tested cytostatic concentration of

213 μM (corresponding to 32-fold TPL). For epithelial cell colony formation, there was a significant correlation ($R^2 > 0.78$) between the HNSCC fragments in respect to their chemoresponse to cisplatin (fig. 2). However, the t -test for paired samples revealed that there were no significant differences ($p > 0.34$).

Cut-off concentrations of docetaxel in colony formation by epithelial cells were in the range of 0.5 to $>17.60 \mu\text{M}$. Also the intratumoral cut-off variation was only marginal (table 2). Cut-offs were identical for all HNSCC fragments in eight cases and varied only one titration step in the other four assays. Colony formation at the highest tested docetaxel concentration ($17.60 \mu\text{M}$) was detectable in all assayed fragments of six HNSCC. Two HNSCC showed this high resistance to docetaxel in one and two of the three tested fragments, respectively. A significant correlation ($R^2 > 0.71$) was found between the separate tumor fragments for epithelial cell colony formation following docetaxel treatment (fig. 2) but significant differences could not be shown in the t -test for paired samples ($p > 0.18$).

Cut-off determination respective to formation of stromal cell colonies

Intertumoral cut-off concentrations of cisplatin for stromal cell colonies were reached in all tests below the highest tested cisplatin concentration of 213 μM . They ranged from 1.67 to $>106 \mu\text{M}$. In contrast to this high intertumoral variability, the intratumoral cut-off concentrations varied only marginally (table 2, fig. 3). This corresponds to the observations in analyses of colony formation by epithelial cells. Cut-off concentrations were identical for all fragments in eight assays and differed in four assays only by one titration step (table 2). There was a significant correlation ($R^2 > 0.98$) regarding the stromal colony formation

between the HNSCC fragments without significant differences in the *t*-test for paired samples ($p>0.17$).

In regard to docetaxel, the intertumoral range of cut-off concentrations for stromal cell colonies was 1.10 to 17.60 μM , whereas the variability of intratumoral cut-off concentrations was small. In nine cases, all fragments of a given tumor had identical cut offs, while this parameter varied only by one titration step in the three divergent cases. In seven out of twelve chemoresponse tests the stromal cell colony formation was detectable up to the highest tested docetaxel concentration (17.60 μM). Despite the significant correlation ($R^2>0.55$), the *t*-test for paired samples revealed that following docetaxel treatment there were no significant differences between cut-off values of the three fragments ($p>0.17$).

Comparison of the cut-off concentrations in formation of epithelial and stromal cell colonies

Chemoresponse-assays with absence of either epithelial or stromal colonies in control wells were not included in this comparison. Consequently, 69 out of 90 single fragments (76.7%) from the total 15 tested HNSCC specimens were evaluable for differences respective to cut-off concentrations of both chemotherapeutics regarding formation of epithelial and stromal cell colonies.

For cisplatin, suppressive activity regarding colony formation of epithelial and stromal cells was different in general. Thus no significant correlation was found in regard to the colony-formation ability of the epithelial and stromal cells (fig. 4).

The cut-off concentrations of docetaxel respective to colony formation of either epithelial or stromal cells were found to be more often identical within one individual HNSCC fragment. Contrary, the cut-off values showed a much higher variability following cisplatin treatment (table 2, fig. 4) and also varied between the fragments of the same HNSCC more than one titration step. This outcome was more frequently observed in response to cisplatin (44.1%) than to docetaxel treatment (only 20% of the cases; table 3). Furthermore, it could be demonstrated that altogether 18.9% of the HNSCC showed an elevated chemoresistance of formation of stromal colonies in comparison to those of epithelial colonies. Noticeable, also the share of high resistant stroma cells was higher in treatment by cisplatin than following administration of docetaxel (26.4% vs. 11.4%). Thus the chemoresponse of epithelial and stromal cell was found to be more homogeneous regarding docetaxel treatment than in treatment by cisplatin (table 2 and 3, figs. 4 and 5). However, a significant correlation of cut-off concentrations of docetaxel regarding colony formation of epithelial and stromal cells of HNSCC was never found (figs. 5).

Discussion

In this study the existence of high chemoresistant stromal cell colonies in a high number of HNSCC and in particular a different chemoresistance of epithelial and stromal cell colonies to chemotherapeutics is demonstrated. This was found for docetaxel and in principal for cisplatin and is belayed to be statistically significant respective to the here presented data for colony-formation of HNSCC *ex vivo*.

HNSCC consist *in vivo* of epithelial cells and non-epithelial (*i.e.* stromal) cells in different quantities. Since both cell types show differences regarding proliferation and growth in dependence on various factors (growth factors, cytokines etc.) present in their environment [28], there is not only a great inter- but also a high intratumoral morphological heterogeneity to be found. This morphological heterogeneity of a tumor *in vivo* is found just as well in the tumor growth *in vitro* [29].

In the study of Wichmann and colleagues [17] only in regard to epithelial cells the representativeness of the chemoresponse of a single biopsy for the whole given HNSCC was emphasized. But since it is very desirable that not only epithelial but all types of cells and in particular the major cell types, the epithelial and stromal cells, proliferate and grow in the *ex-vivo* chemoresponse assay in an *in-vivo* like manner, it is also important to examine all cell populations separately regarding their response to chemotherapeutical treatments. This is necessary to avoid misinterpretation due to limited data basis and to reach a conclusion whether or not the chemoresponse of one cell type allows inferences respective to the chemoresponse of all other cell types. Only such a complete approach allows for the translation of data gained *ex-vivo* into the situation *in vivo*.

The used *ex-vivo* chemoresponse tests as well as the concomitant KB-cell experiment for verification of the test's reproducibility were carried out under flavin-protecting conditions to avoid flavin-mediated photoreactions [15, 16]. As demonstrated by the 14 KB-cell tests, which in general confirmed the predetermined IC₅₀ values of cisplatin and docetaxel [24, 25], the flawless reproducibility has been proven. Hence there is no denying that the execution of all assays was valid. Thus it might as well be concluded that this is true also for the culturing of the major cell populations present in HNSCC within the *ex-vivo*

assay. Therefore the tests indeed allow for a detailed analysis of colony formation by epithelial and stromal cell under exposure to either cisplatin or docetaxel. Comparing this test method with two well-known chemoresponse assays – the EDR and ATP-TCA assays – exposes some flaws in both of the established test systems. The EDR assay only allows statements to the chemoresistance of a malignant tumor, it does not allow statements about the patient's individually best fitting chemotherapeutic agents for a sufficient therapy of its malignancy and thus has only limited value respective to detection of a suitable treatment. Especially against the background of the increasing number of new cytostatic agents including small molecules and biologicals, a statement about the chemosensitivity of a cancer – and so about a probably effective therapy option – is more preferable for decision-making in head and neck oncology than the declaration of high chemoresistance of tumors. Besides, due to the cooling of the tumor material during the shipping before testing, and in particular the usage of medium with high riboflavin-concentrations and the handling under normal illumination, phototoxic reactions may occur and influence the test results leading to chaotropism and unpredictability of chemoresponses of the malignancy to tolerable doses *in vivo*. Besides the limitations mentioned above the ATP-TCA uses trypsination for digestion which results in a suspension of singularized cells. Thus, the interaction between epithelial and stromal cells as well as their different chemoresponse is not reflected properly and results *in vitro* basically and essentially must be deviant from those *in vivo* since solid tumors in no way represent a singularized cell suspension. However, the measurement of sum signals reflecting colony formation, proliferation or cytotoxicity, and use of such measurements can not be suitable for evaluation of the chemoresponse of a HNSCC. In particular, and as shown by our data, definite findings are not deducible about the chemoreactivity of a malignancy, and congruence in chemoresponse *in vitro* and *in vivo* will be hardly achieved whenever only sum signals are measured. In the used *ex-vivo* chemoresponses assay at least the major cell types present in HNSCC can be differentiated using Giemsa staining. Hence colonies of fibrocytes, endothelial and epithelial cells were clearly distinguishable (fig. 1)

based on the described morphology and the efficiency of the chemotherapeutics can be examined. To minimize possible mistakes in interpretation of microscopic observations and in particular to achieve proper differentiation between colonies of epithelial and stromal cells that were formed during *ex-vivo* treatment, and especially taking into account their somewhat irregular morphological appearance that may be leading to misinterpretation by students and even pathologists without experience, the counting was carried out by two well-trained investigators independently. To allow in the future for an easier differentiation of all cell types and as well an analysis of the exact composition of the stromal colonies, new studies should include marker analyses using fluorescent-labelled antibodies. But despite possible advantages due to the use of sufficient markers and their labelling by fluorescent dyes allowing also differentiation of cell populations by the untrained, a correct evaluation of the outcome in *ex-vivo* testing is achievable also by use of Giemsa staining (fig. 1). As shown here, the necessary dose of cytostatics to achieve suppression of colony formation varies strongly dependent on the cytostatics used and the cell type examined. Regarding the total spectrum of the 15 HNSCC tested in this study, the spectra of cut-off concentration for cisplatin were 3.3 to 53.3 μM for epithelial and 1.7 to 106.7 μM for stromal cell colonies. For docetaxel the cut-off was between 0.5 and to 17.6 μM for epithelial and 1.1 and 17.6 μM for stromal cell colonies.

In contrast to the great pharmacological heterogeneity of the 15 HNSCC tested, the chemoresponse of the three specimens of a given tumor was quite homogeneous. This homogeneity in general is the case in comparisons made

for colonies of the same major cell population, either epithelial or stromal cells. It was found that the cut-off concentration of three fragments of a HNSCC varied for each of the particular cell population only marginally (about one titration step at most). This was regarded to be assay-related fluctuation due to statistical reasons. Hence, there was a significant correlation between the three specimens of the individual HNSCC. Moreover, no statistical difference was found applying the *t*-test for paired samples, neither in the treatment with cisplatin (epithelial colonies: $p>0.34$; stromal colonies: $p>0.17$) nor docetaxel ($p>0.18$ and $p>0.17$, respectively).

Comparing cut-off concentrations of the epithelial and stromal colonies within a fragment it was noticed that in 18.9% of all assays (and accordingly 26.4% of the assays with cisplatin and 11.4% with docetaxel) the cut-off concentration for the stromal colony formation within a fragment was considerably higher (>2 titration steps) than the cut-off for epithelial cell colonies (table 3). *Vice versa* higher resistance of epithelial cells was found only in 13% of all assays (accordingly 17.7% with cisplatin and 8.6% with docetaxel). This more often found resistance of stromal cells in comparison to epithelial cells is reflected by the graphs shown in figure 3. Such a higher resistance of stromal cells was also described as outcome in *ex-vivo* chemoresponse assays for HNSCC [25, 30] and lung cancer [31]. The chemoreactivity of epithelial and stromal cell was more homogeneous regarding treatment with docetaxel than with cisplatin. Thus, there was no significant correlation of colony formation ability of epithelial and stromal cells for either cisplatin or docetaxel. These results demonstrate that the used *ex-vivo* chemoresponse assay is able to discover a distinct

pharmacological behavior or, in other words, heterogeneity in colony formation ability of different cell types treated with cytostatics. Moreover, it underlines that errors are unavoidable if the evaluation of chemoresponses based on cut-off concentrations does not differentiate between the major cell populations. In addition, there is a much higher homogeneity within the HNSCC specimens regarding the outcome of chemoresponse testing in separate analysis of each cell population. This – as already stated by Wichmann and colleagues [17] for epithelial cell colonies – allows for estimation of the chemoresponse of the total HNSCC respective to a particular (but as shown here: only to the *one* particular) cell population analyzed.

Therefore, an analysis of sum responses for the evaluation of the chemoresponse of HNSCC, like commonly used in most of the publications [e.g. 32, 33], appears to be obsolete, because misinterpretation of the results might be possible due to the occurrence of high resistant stromal cells and a predominant lack of correlation between colony formation of epithelial and stromal cells.

Up to date the cause of the more often observed increased chemoresistance of stromal cells is not resolved conclusively. It is known that fibroblast and fibrocytes affect tumor growth, progression, invasion and resistance to chemotherapeutics over autocrine and paracrine signal pathways [34-37]. To analyze the various cell types in HNSCC regarding their potentially different chemoresponse *ex vivo* and to include also the knowledge about interaction of these cells into interpretation of the *ex-vivo* data might be an efficient way to better predict the response of an individual HNSCC to a treatment *in vivo*.

Conclusion

The existence of highly chemoresistant stromal cell colonies in some HNSCC and in particular a difference in the chemoresponse of epithelial and stromal cells within a given HNSCC to either cisplatin or docetaxel was demonstrated in this study. Consequently the separate analysis of epithelial and stromal cell colony formation is necessary for sufficient evaluation of chemoresponses of HNSCC in an *ex-vivo* assay to allow adequate predictive statements about the tumor's chemoreactivity *in vivo*.

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Tab.1: Age, gender, localization and stadium of disease respective to AJCC criteria of patients included in the study and TNM-classification of the HNSCC of which three tissue specimens were investigated in the *ex-vivo* chemoresponse test.

Tab. 2: Cut-off concentrations (concentrations of either cisplatin or docetaxel required to suppress formation of colonies) for three tissue samples of individual HNSCC respective to colonies of epithelial and stromal origin.

Tab. 3: Comparative overview on the cut-off concentrations in suppression of formation of epithelial and stromal cell colonies by cisplatin and docetaxel.

Fig. 1: Giemsa-stained colonies of a HNSCC cultured three days in the flavin-protecting *ex-vivo* colony formation assay. A: epithelial cell colonies. B & C: stromal cell colonies consisting of either fibrocytes (B) or endothelial cells (C). D: mixed colonies (simultaneous appearance of epithelial and stromal colonies in close proximity) Bar = 100 μ M.

Fig. 2: Correlation of cut-off concentrations in formation of epithelial cell colonies of the three fragments of the 12 HNSCC fulfilling inclusion criteria for cut-off determination. A-C: cut-off concentrations of cisplatin. D-F: cut-off concentrations of docetaxel. As shown by the coefficient of determination (R^2), a significant correlation between cut-off concentrations of the three fragments is generally found. If identical cut-off concentrations of two or more HNSCC were detected, the quantity of data points on the same coordinates is inserted in Arabic numbers.

Fig. 3: Correlation of cut-off concentrations in formation of stromal cell colonies of the three fragments of the 12 HNSCC fulfilling inclusion criteria for cut-off determination. A-C: cut-off concentrations of cisplatin. D-F: cut-off concentrations of docetaxel. As shown by the coefficient of determination (R^2), a significant correlation between cut-off concentrations of the three fragments is generally found. If identical cut-off concentrations of two or more HNSCC were detected, the quantity of data points on the same coordinates is inserted in Arabic numbers.

Figure 4: Correlation of cut-off concentrations of cisplatin in formation of epithelial (abscissa) and stromal cell colonies (ordinate) of the three fragments of the 12 HNSCC fulfilling inclusion criteria for cut-off determination. A-C: Cut-off concentrations for epithelial cells of fragment 1 and stromal cells in the three fragments. D-F: Cut-off concentrations for epithelial cells of fragment 2 and stromal cells in the three fragments. G-I: Cut-off concentrations for epithelial cells of fragment 3 and stromal cells in the three fragments. As shown by the coefficient of determination (R^2) a significant correlation between cut-off concentrations of the three fragments is absent. If identical cut-off concentrations of two or more HNSCC were detected, the quantity of data points on the same coordinates is inserted in Arabic numbers.

Figure 5: Correlation of cut-off concentrations of docetaxel in formation of epithelial (abscissa) and stromal cell colonies (ordinate) of the three fragments of the 12 HNSCC fulfilling inclusion criteria for cut-off determination. A-C: Cut-off concentrations for epithelial cells of fragment 1 and stromal cells in the three fragments. D-F: Cut-off concentrations for epithelial cells of fragment 2 and stromal cells in the three fragments. G-I: Cut-off concentrations for epithelial cells of fragment 3 and stromal cells in the three fragments. As shown by the coefficient of determination (R^2) no significant correlation between cut-off concentrations of the three fragments is found. If identical cut-off concentrations of two or more HNSCC were detected, the quantity of data points on the same coordinates is inserted in Arabic numbers.

Tab. 1.

Pat.-ID	Age	Gender	Localization of HNSCC	Stadium (AJCC)	TNM classification
1	58	Male	Oral cavity	I	T ₁ N ₀ M ₀
2	62	Male	Oropharynx	IVa	T ₂ N _{2b} M ₀
3	57	Male	Oropharynx	IVa	T ₂ N _{2b} M ₀
4	52	Female	Oropharynx	IVa	T ₂ N _{2b} M ₀
5	42	Male	Larynx	IVb	T _{4b} N _{2c} M ₀
6	45	Male	Oropharynx	IVa	T ₂ N _{2b} M ₀
7	65	Male	Oropharynx	IVa	T ₂ N _{2b} M ₀
8	50	Male	Oropharynx	III	T ₃ N ₁ M ₀
9	53	Male	Oropharynx	IVa	T ₁ N _{2b} M ₀
10	71	Male	Hypopharynx	III	T ₃ N ₀ M ₀
11	50	Male	Oral cavity	II	T ₂ N ₀ M ₀
12	52	Male	Oropharynx	II	T ₂ N ₀ M ₀
13	31	Male	Sphenoid bone	IVa	T _{4a} N ₀ M ₀
14	58	Male	Larynx	IVa	T _{4a} N _{2c} M ₀

Tab. 2.

Pat.-ID	fragment	Cut-off concentration for epithelial colonies [μM]		Cut-off concentration for stromal colonies [μM]	
		cisplatin	docetaxel	cisplatin	docetaxel
01	01-1	53.33	0.55	13.33	8.80
	01-2	53.33	1.10	13.33	8.80
	01-3	53.33	0.55	13.33	8.80
02	02-1	26.67	8.80	1.67	8.80
	02-2	26.67	8.80	1.67	8.80
	02-3	53.33	8.80	1.67	8.80
03	03-1	3.33	4.40	13.33	17.60
	03-2	3.33	4.40	13.33	8.80
	03-3	6.67	4.40	13.33	8.80
04	04-1	13.33	17.60	13.33	17.60
	04-2	13.33	17.60	13.33	17.60
	04-3	13.33	17.60	6.67	17.60
06	06-1	13.33	17.60	106.67	17.60
	06-2	13.33	17.60	106.67	17.60
	06-3	13.33	17.60	106.67	17.60
07	07-1	13.33	17.60	26.67	17.60
	07-2	6.67	17.60	26.67	17.60
	07-3	6.67	17.60	26.67	17.60
09	09-1	13.33	17.60	26.67	17.60
	09-2	13.33	17.60	26.67	17.60
	09-3	6.67	17.60	26.67	17.60
10	10-1	6.67	17.60	6.67	8.80
	10-2	6.67	17.60	13.33	8.80
	10-3	6.67	17.60	13.33	8.80
11	11-1	13.33	8.80	13.33	1.10
	11-2	13.33	8.80	6.67	1.10
	11-3	13.33	8.80	6.67	1.10
12	12-1	13.33	8.80	13.33	8.80
	12-2	13.33	8.80	13.33	8.80
	12-3	13.33	17.60	6.67	8.80
13	13-1	3.33	17.60	n.e.	17.60
	13-2	3.33	8.80	3.33	8.80
	13-3	3.33	17.60	3.33	17.60
14	14-1	26.67	17.60	13.33	17.60
	14-2	26.67	17.60	13.33	17.60
	14-3	n.e.	n.e.	13.33	8.80

Tab. 3

Chemotherapeutic Agent	Number of tissue samples*	Cut-off concentration C					
		$C_{\text{epithelial}} > C_{\text{stromal}}$		$C_{\text{epithelial}} = C_{\text{stromal}}$		$C_{\text{epithelial}} < C_{\text{stromal}}$	
		N	[%]	N	[%]	N	[%]
Cisplatin	34	06	17.7	19	55.9	09	26.4
Docetaxel	35	03	08.6	28	80.0	04	11.4
Sum	69	09	13.0	47	68.1	13	18.9

* The number given represents the number of tissue specimens fulfilling inclusion criteria regarding formation of at least two colonies of both types of cells differentiated.

Fig. 1.

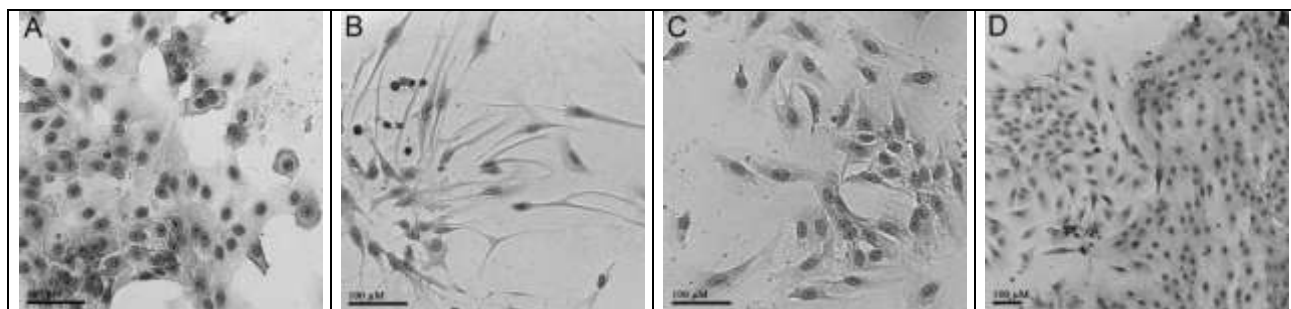


Fig. 2

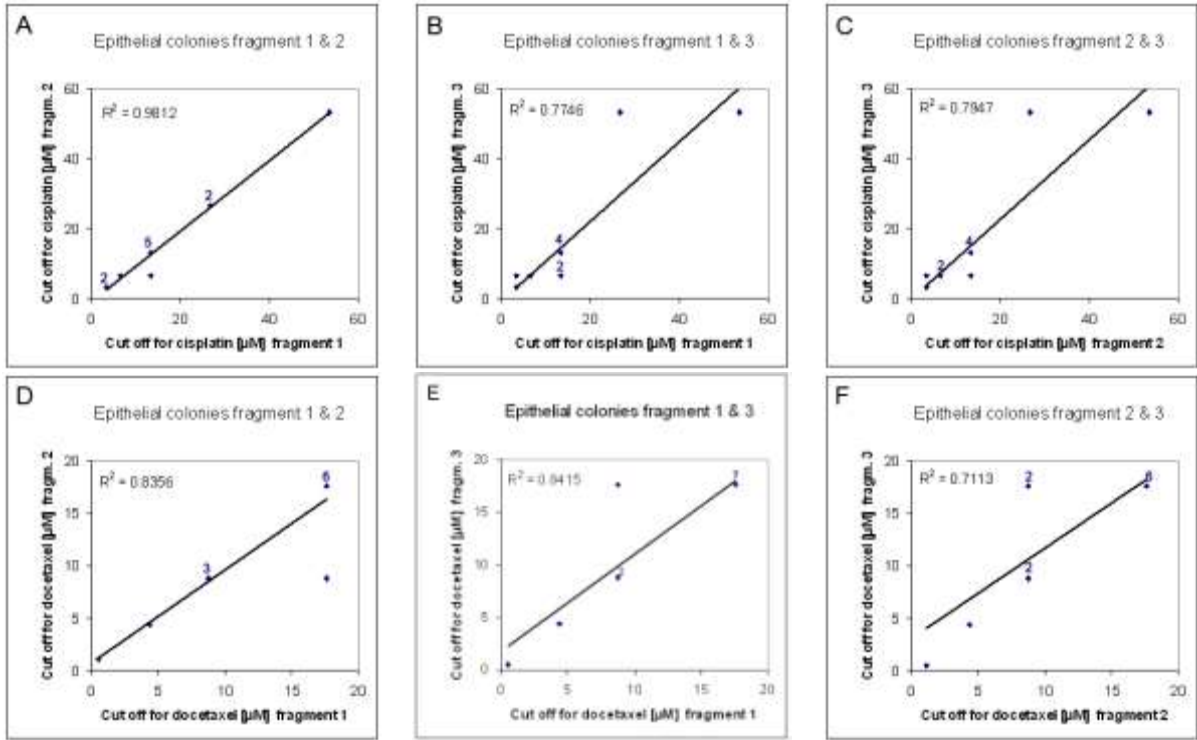


Fig. 3

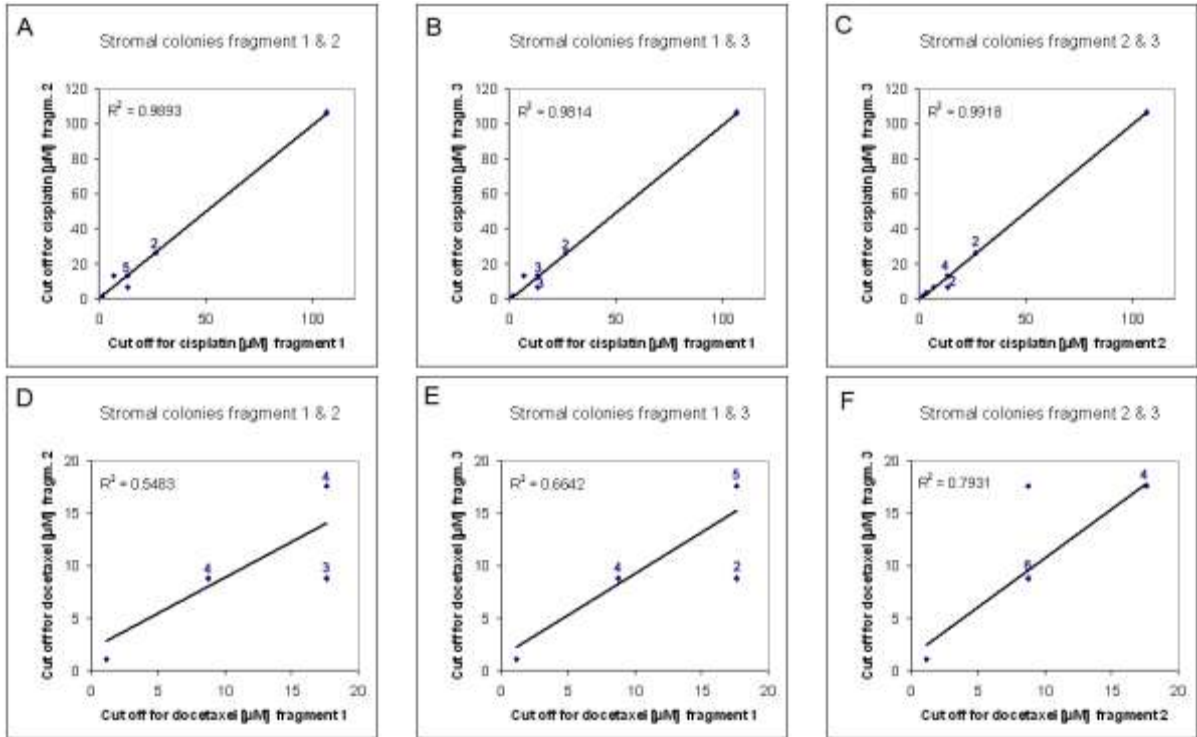


Fig. 4.

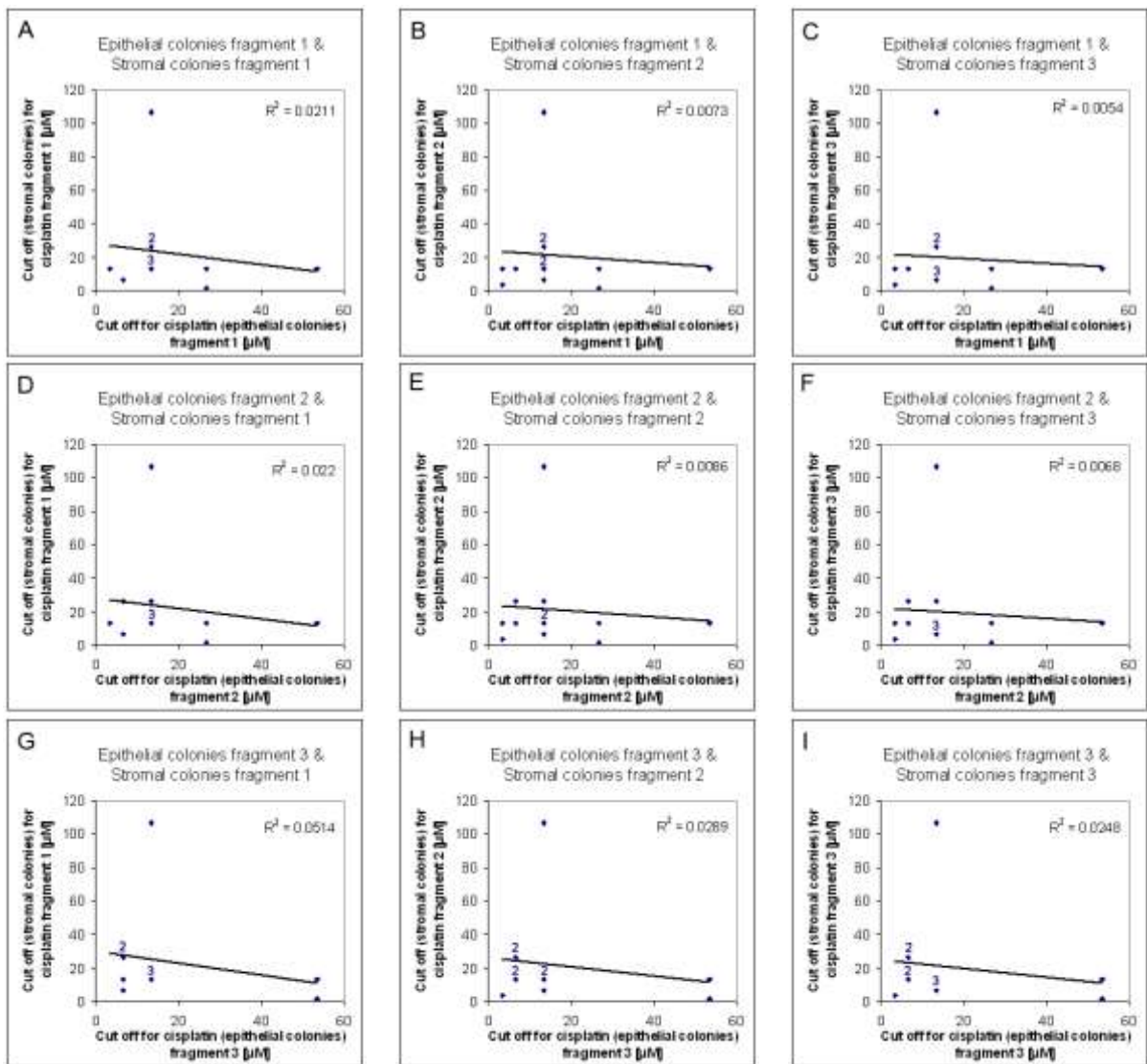


Fig. 5.

