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# Slice cultures from head and neck squamous cell carcinoma: a novel test system for drug susceptibility and mechanisms of resistance

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**Background:** Human head and neck squamous cell carcinoma (HNSCC) fundamentally vary in their susceptibility to different cytotoxic drugs and treatment modalities. There is at present no clinically accepted test system to predict the most effective therapy for an individual patient.

**Methods:** Therefore, we established tumour-derived slice cultures which can be kept *in vitro* for at least 6 days. Upon treatment with cisplatin, docetaxel and cetuximab, slices were fixed and paraffin sections were cut for histopathological analysis.

**Results:** Apoptotic fragmentation, activation of caspase 3, and cell loss were observed in treated tumour slices. Counts of nuclei per field in untreated compared with treated slices deriving from the same tumour allowed estimation of the anti-neoplastic activity of individual drugs on an individual tumour.

**Conclusion:** HNSCC-derived slice cultures survive well *in vitro* and may serve not only to improve personalised therapies but also to detect mechanisms of tumour resistance by harvesting surviving tumour cells after treatment.

Treatment of human head and neck squamous cell carcinoma (HNSCC) imposes a major challenge for clinicians and patients, as primary surgery, especially in advanced stages, can cause severe disability, while chemotherapy and irradiation can lead to dreadful early and late toxicity. Secondary surgery can be extremely difficult due to irradiation-induced tissue alterations but becomes necessary if patients do not respond to primary chemo-radiation (Boehm *et al*, 2010; Dietz *et al*, 2012). At present, there is no established test system allowing for prediction of whether or not an individual tumour responds to chemotherapy and/or irradiation. Therefore, current decision making is based on randomised clinical trials which – by their nature – are designed to statistically evaluate the response within a total cohort rather than the response of individual patients.

To bypass this conflict, an ongoing trial (DeLOSII) uses the tumour response after short induction chemotherapy as assumed indication of susceptibility for the planned therapeutic protocol. In addition, we have exposed tumour-derived cells cultured under flavin-free conditions (FLAVINO assay) to cytotoxic drugs in an attempt to test their efficiency *ex vivo* (Dollner *et al*, 2004; Dietz *et al*, 2010; Wichmann and Dietz, 2011; Wichmann *et al*, 2011; Schrader *et al*, 2012). Using this system, we demonstrated differences in the response of epithelial vs stromal cells in HNSCC (Horn *et al*, 2010). As extracellular matrix (ECM) epitopes, which are lost in cell cultures, provide important signals for cell differentiation and survival (Schaefer *et al*, 2005; Babelova *et al*, 2009), we now established a protocol to culture organotypic slices from HNSCC and tested their response to established cytotoxic

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drugs (cisplatin, docetaxel and cetuximab) currently used in standardised treatment concepts for HNSCC.

Tumour-derived organotypic slice cultures provide a unique tool to study tissue responses to various stimuli, including drugs and irradiation (Nitsch *et al*, 2000; Merz *et al*, 2010; Rajendran *et al*, 2010; Vaira *et al*, 2010; Merz and Bechmann, 2011; Holliday *et al*, 2013). The open system allows direct access and the option to collect samples from media over time. At the end of the experiment, slices can be embedded in paraffin for histological analysis using for example, immune cytochemistry for the detection of DNA damage after irradiation ( $\gamma$ H2AX), apoptosis (activated caspase 3), or proliferation (Ki-67) (Merz *et al*, 2013). Here, we show that HNSCC-derived slices survive well in culture

for up to at least 6 days and exhibit the expected cell loss upon treatment with cytotoxic drugs rendering them a promising tool to predict tumour responses and to study mechanisms of tumour resistance.

## MATERIALS AND METHODS

**Tumour slice preparation.** Informed consent was obtained from all patients, and the investigation was approved by the ethics committee of the Medical Faculty of the University Leipzig (votes No. 201-10-12072010 and No. 202-10-1207210). Tissue was obtained from patients during resection of HNSCC (tonsils, base

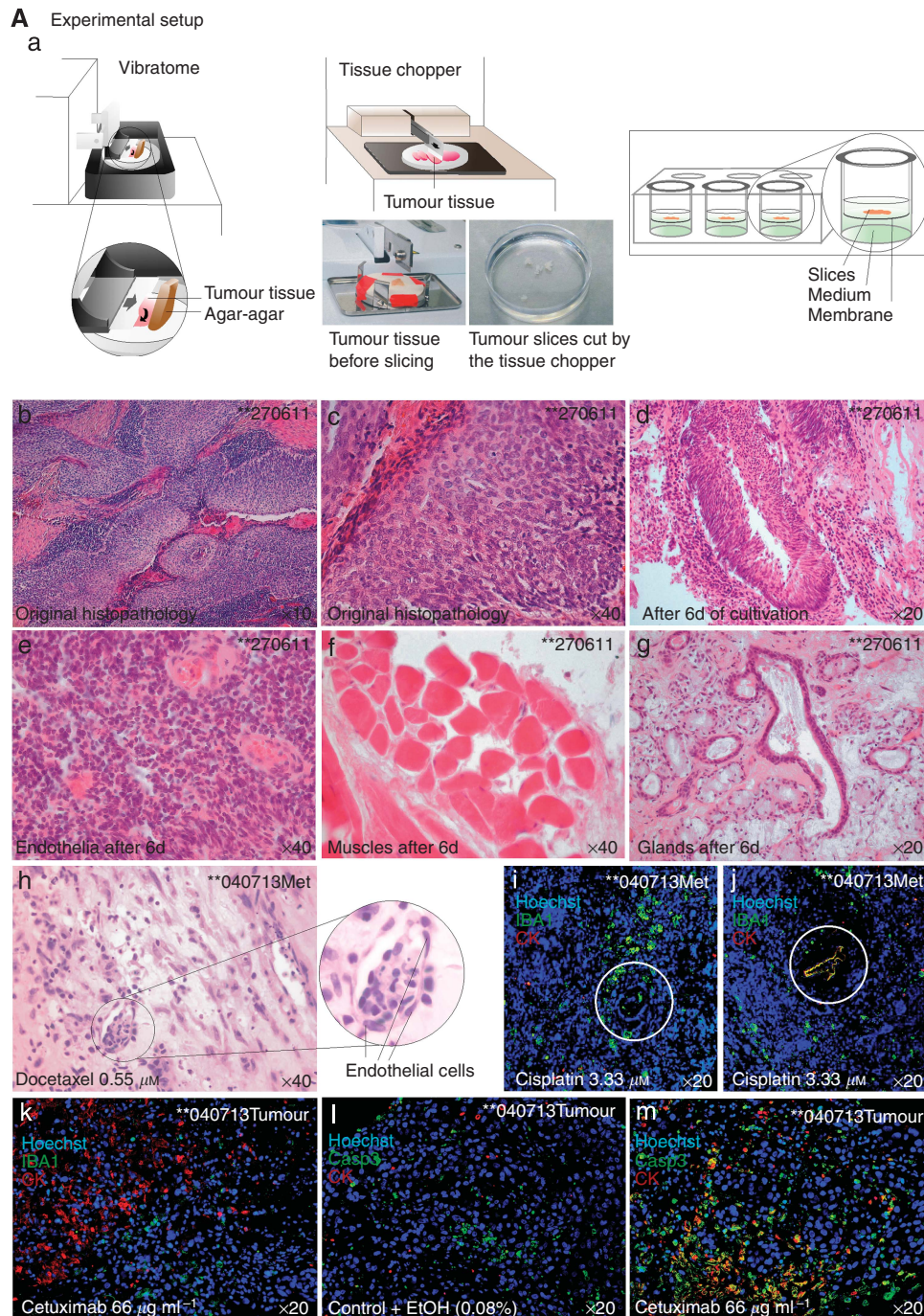


Figure 1. (Continued)

of tongue, larynx, pharynx or metastases in cervical lymph nodes) at the Clinic of Otorhinolaryngology, University Hospital Leipzig, Leipzig, Germany. The specimens for experiments were kept in tumour medium (TM, please see below) and transported to the lab, where they were put into an incubator until further preparation. In parallel, the tumour and its histology was classified by a board certified pathologist (CW). Tissue slices of 350  $\mu\text{m}$  were prepared

under sterile conditions either using a vibratome (Leica VT 1000, Leica Microsystems GmbH, Wetzlar, Germany) or a tissue chopper (McIlwain TC 752; Campden Instruments, Lafayette, IL, USA) between 3 and 48 h after surgery. Slices were then placed on the membrane culture inserts (Millipore Corporation, Billerica, MA, USA) in six-well-plates containing TM. TM is a custom-designed phenol red and riboflavin-free cultivation medium (RPMI 1640;

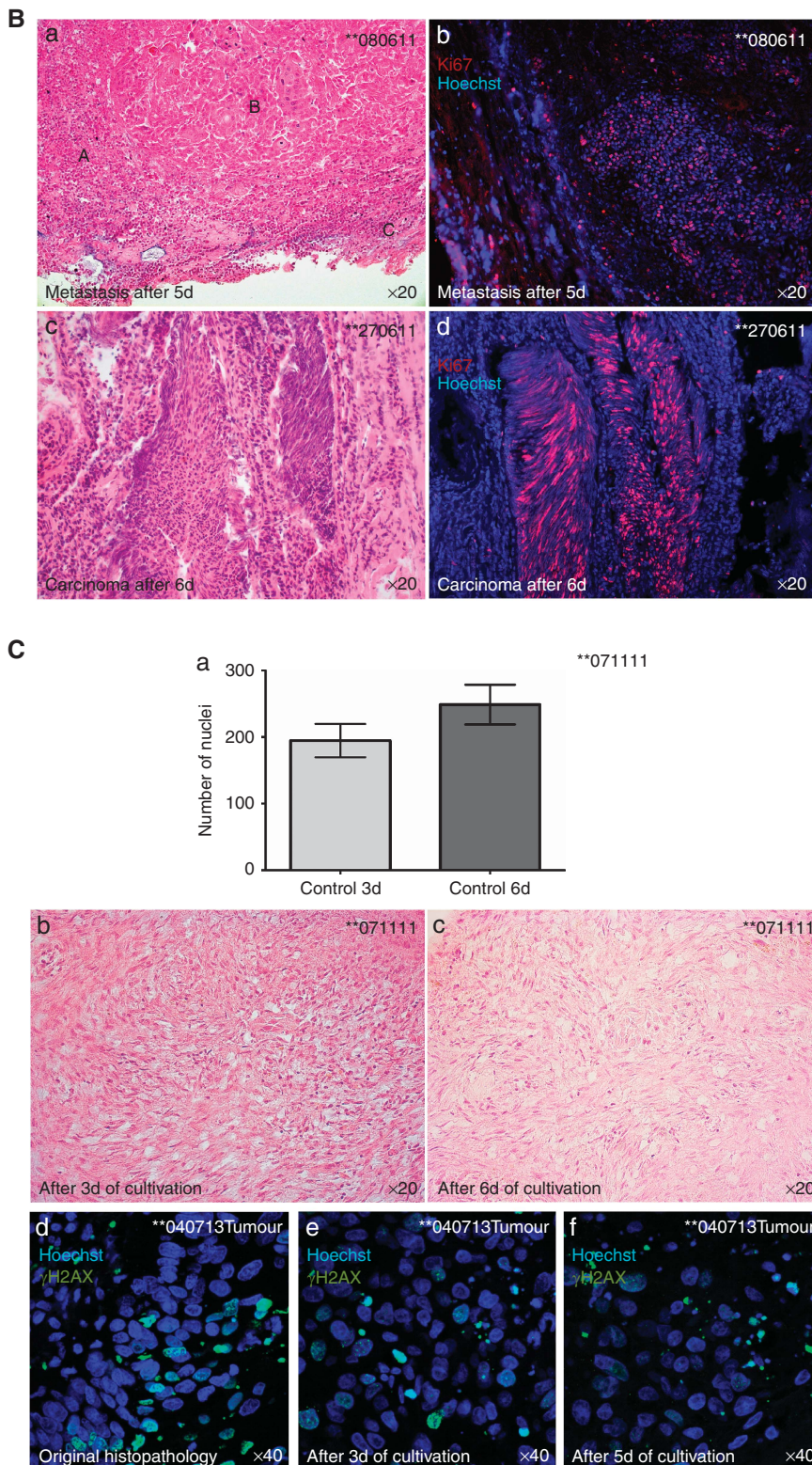


Figure 1. See caption for next page

Bio & Sell, Feucht, Germany) consisting of  $1.134\text{ g l}^{-1}$   $\text{NaHCO}_3$ ,  $14.5\text{ mM}$  HEPES and  $20\text{ mg l}^{-1}$  l-cystine. TM was supplemented before use with 10% (v/v) fetal calf serum (FCS; Invitrogen, Darmstadt, Germany), amikacin, nystatin, penicillin and streptomycin (all from Sigma, Munich, Germany) and underwent sterile filtration ( $0.22\text{ }\mu\text{M}$ ). Following the addition of FCS, TM contained  $20\text{ nM}$  of riboflavin. Later, slices were cultivated in a humidified incubator at 5%  $\text{CO}_2$  and  $37\text{ }^\circ\text{C}$ . Medium was changed at least two times a week.

**Cytotoxic treatment of slice cultures.** For treatment of slices, cisplatin ((SP-4-2)-Diamindichloridoplatin(II); DDP; Sigma), docetaxel (Taxotere;  $20\text{ mg ml}^{-1}$ ; Sanofi-Aventis Deutschland GmbH, Berlin, Germany) and cetuximab (Erbix;  $5\text{ mg ml}^{-1}$ ; Merck Serono, Munich, Germany) were used. Cisplatin was dissolved in sterile  $\text{H}_2\text{O}$  and diluted in culture medium to final concentrations of  $6.66$  and  $3.33\text{ }\mu\text{M}$ . Before testing docetaxel in its final concentrations of  $0.55$  and  $0.275\text{ }\mu\text{M}$ , it was dissolved in ethanol and diluted in culture medium. Cetuximab was tested in a concentration of  $66\text{ }\mu\text{g ml}^{-1}$  and diluted in TM. The incubation period was up to 7 days, and medium was changed at least two times with newly prepared dilutions of drugs. After the treatment, slices were fixed and processed as described below.

**Staining procedure for morphological analysis.** After a minimum of 5 h and a maximum of 7 days, the tumour slices were fixed in paraformaldehyde (4%), washed with phosphate-buffered saline (PBS) and embedded in paraffin. Before staining with hematoxylin/eosin (HE) for histology, sections were cut on a microtome ( $4\text{--}7\text{ }\mu\text{m}$ ). In order to perform immunohistochemical stainings, paraffin sections were dewaxed in xylene, rehydrated in graded alcohol and consecutively retrieved in citrate buffer at  $96\text{ }^\circ\text{C}$  for 10 min in a microwave. Sections were then rinsed in PBS/Triton (0.3%) and blocked with 5% normal goat serum in 0.3% PBS/Triton for 1 h. The primary antibodies against Ki-67 (Becton Dickinson, Franklin Lakes, NJ, USA; mouse, 1:200), cleaved caspase 3 (Cell Signaling Technology, Inc., Danvers, MA, USA; rabbit, 1:400), IBA1 (Wako Chemicals GmbH, Neuss, Germany; rabbit, 1:400), cytokeratin (Dako Denmark A/S, Glostrup, Denmark; Clone D5/16 B4, mouse, 1:150) and  $\gamma\text{H2AX}$  (Millipore, mouse, 1:100) were allowed to incubate overnight at  $4\text{ }^\circ\text{C}$ . Sections were then rinsed again with 0.3% PBS/Triton and incubated with appropriate fluorescent-labelled secondary antibodies (goat-anti-mouse, goat-anti-rabbit, Alexa Fluor 568 and 488) and Hoechst 33342 for visualisation of nuclei. Images were taken and analysed

using an Olympus BX51 fluorescence microscope (Olympus Europa Holding GmbH, Hamburg, Germany) or a Zeiss LSM 510 confocal microscope (Carl Zeiss AG, Oberkochen, Germany) for fluorescent staining and a Zeiss Axioplan 2 (Carl Zeiss AG) for HE staining.

**Quantification of nuclei and analysis of cell death.** Total cell numbers of sections were determined by counting nuclei in HE-stained slices with the Image J plug-in Cell Counter (NIH, Bethesda, MD, USA). Apoptosis was also determined using Cell Counter by identifying caspase-3-positive cells in relation to total cell number (Hoechst positive). Pictures taken at a magnification of  $\times 400$  were analysed to this end. Statistics were calculated with Graph Pad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) (*t*-test for two or one-way ANOVA for more groups;  $P < 0.05$  was considered significant).

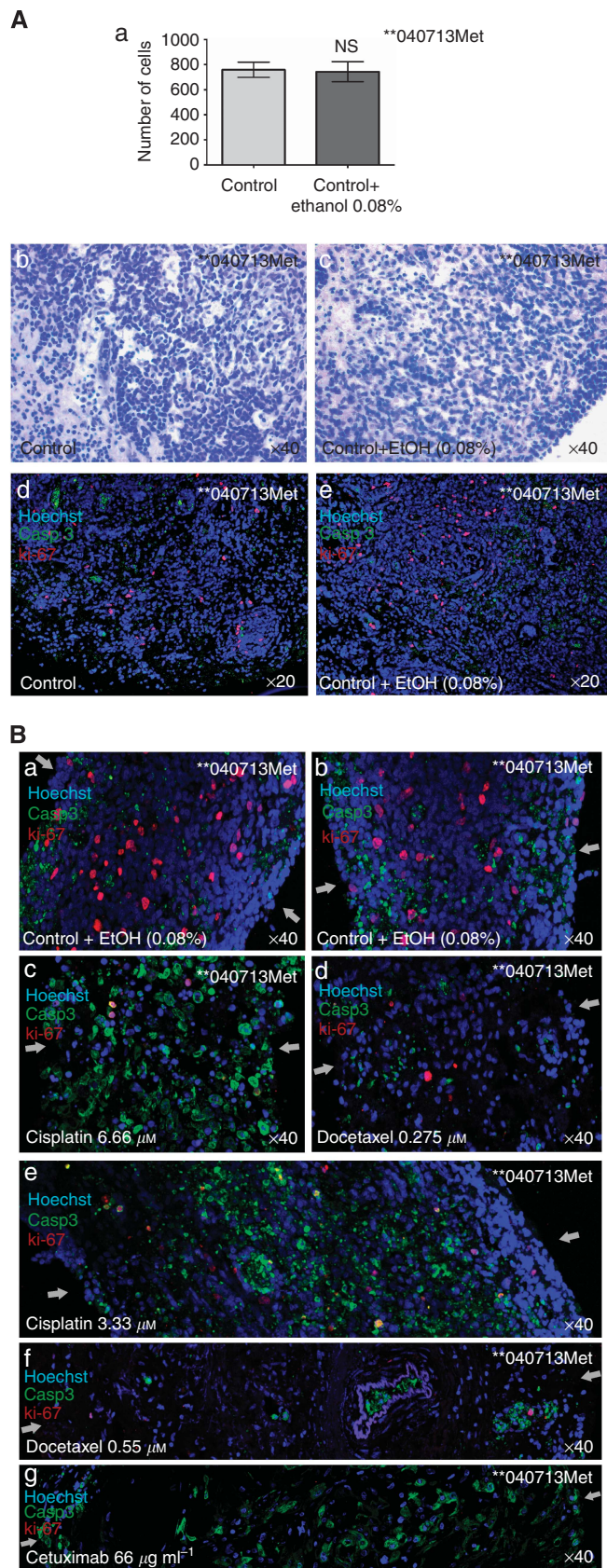
## RESULTS

**Slice culture of HNSCC.** Tumour tissues obtained from patients after surgical resection were cut using a vibratome (Leica VT1000) or a tissue chopper (McIlwain TC 752) at a thickness of  $350\text{ }\mu\text{m}$  and transferred onto membrane inserts (Millipore) within six-well plates for further cultivation (Figure 1Aa). Owing to the texture of the tissue, some tumours could not be cut properly with a vibratome or consecutive sections revealed different thicknesses, whereas cutting with a tissue chopper provided better and more precise results.

For morphological analysis, HE stainings were performed on paraffin sections of tissue slices after a cultivation period of 5 h to 7 days. Slice cultures were viable and maintained their typical morphological features for up to 6 days *in vitro* (Figure 1Ad) as compared to the original diagnostic histopathology (Figures 1Ab and 1Ac). After 7 days, tissue quality was found to suffer in some tumour slices (not shown). Quantification of Hoechst-positive nuclei (Figure 1Ca) and morphological analysis of slices fixed at different time points within 6 days (Figures 1Cb and 1Cc) did not reveal severe tissue alterations within this period of time. Non-tumour cells, for example, endothelia (Figures 1Ae and 1Ah), striated muscle cells (Figure 1Af) and glands (Figure 1Ag), revealed excellent preservation even after treatment with cytotoxic drugs (Figure 1Ah). In addition to morphological analysis by HE staining (Figures 1Ba, Bc Cb and Cc; Figures 2Ab and Ac; Figure 3;

**Figure 1. Preparation, cultivation and survival of slice cultures of HNSCC.** (A) Experimental setup and histopathology. (a) At first, tumour tissue was cut using a vibratome. This technique sometimes provided problems because of the tissue's tendency to slip away and its heterogeneous structure. In contrast, using a tissue chopper, it is possible to reproducibly cut  $350\text{-}\mu\text{m}$  thick slices, which can be cultivated on membrane inserts in six-well plates such that the surface of the media contacts the membrane enabling diffusion. The open system allows easy access for treatment and harvesting of media or parts of the slice. (b, c) Original histopathology of a squamous cell carcinoma of the tonsil, HE-stained. (d, e) HE-paraffin sections of the same tumour after 6 days *ex vivo*. All structures maintained excellent preservation and showed the typical growth pattern of a poorly differentiated squamous cell carcinoma of the tonsil. (f, g) Surrounding tissues such as stratified muscle cells and glands remained vital during the process of cultivation of nearly 1 week. (h–j) Slices of a metastasis of HNSCC after 3 days of cultivation, HE-stained (h) and antibody-labelled for IBA1 (green) and cytokeratin (red) (i, j). Even after treatment with different cytotoxic drugs for 2 days, endothelial cells survive and do not seem to suffer from cytotoxic side effects. (k) In order to investigate the cellular composition of the tissue, IBA1-positive immune cells (green) can be found next to cytokeratin-positive tumour cells (red). (l, m) Increased activation of caspase 3 in tumour cells after treatment with cytotoxic drugs compared with untreated control is visualised by antibody staining for caspase 3 (green) and cytokeratin (red). (B) Proliferation in untreated tumour-slices. (a, b) Slices of a lymph node metastasis of HNSCC after 5 days *ex vivo*, HE-stained (a) and antibody-labelled for Ki-67 (red) and Hoechst (blue) (b). The lymphatic tissue (A) and the fibrous capsule (C) are still visible and can be distinguished from cells of the infiltrating moderately differentiated squamous cell carcinoma (B) (a). Proliferative activity is still present after 5 days of cultivation and visualised by antibody-staining with Ki-67 (b). (c, d) Carcinoma of the tonsil after 6 days *ex vivo*, HE-stained (c) and antibody-labelled for Ki-67 (red) and Hoechst (blue) (d). After 6 days *in vitro*, the tumour tissue is still intact and vivid proliferation is evident. (C) Histopathology over time. (a) Number of Hoechst-positive nuclei after 3 and 6 days of cultivation. (b, c) HE-stained paraffin sections of a carcinoma of the base of the tongue at days 3 and 6 *ex vivo* without cytotoxic treatment. No distinct morphological differences between day 3 and day 6 can be found. (d–f)  $\gamma\text{H2AX}$ -antibody marking double-strand breaks (green) and nuclear counter-staining with Hoechst (blue). No change of  $\gamma\text{H2AX}$  positivity is visible at any of the tested time points.

Figure 4), evaluation of cytokeratin- or IBA1-positive cells (Figures 1Ai and 1Aj) was performed in order to investigate the cellular composition of the tissue. Cytokeratin-positive cells showed increased expression of activated caspase 3 after treatment



compared with cytokeratin-negative cells and untreated controls (Figures 1Al and 1Am). Proliferation was detected by Ki-67 antibodies and nuclear counterstaining with Hoechst 33342 (Figures 1Bb and 1Bd; Figures 2Ad and 2Ae; Figure 2B). After 5 (Figure 1Bb) and 6 (Figure 1Bd) days *in vitro*, untreated slices maintained a high proliferative activity. With the help of  $\gamma\text{H2AX}$  antibodies, double-strand breaks were visualised in slices before cultivation as well as in slices after 3 and 6 days of cultivation (Figures 1Cd-f).  $\gamma\text{H2AX}$  foci were spread evenly in nuclei of all areas of the slice, and no distinct change was found at any of the analysed time points.

The membrane-supported cultivation technique produces two surfaces of the slice, one connected to the membrane and the medium and the other with contact to the air. Thus, vertical gradients in cell proliferation or apoptotic activity must be considered. Although it is not possible to distinguish between the upper and lower surface once fixation and automated embedding are done, transverse sections of the slices provide no evidence for proliferative or apoptotic gradients but detect evenly activated caspase 3 throughout the treated slices rather than showing predominant appearance at the surfaces (Figure 2B).

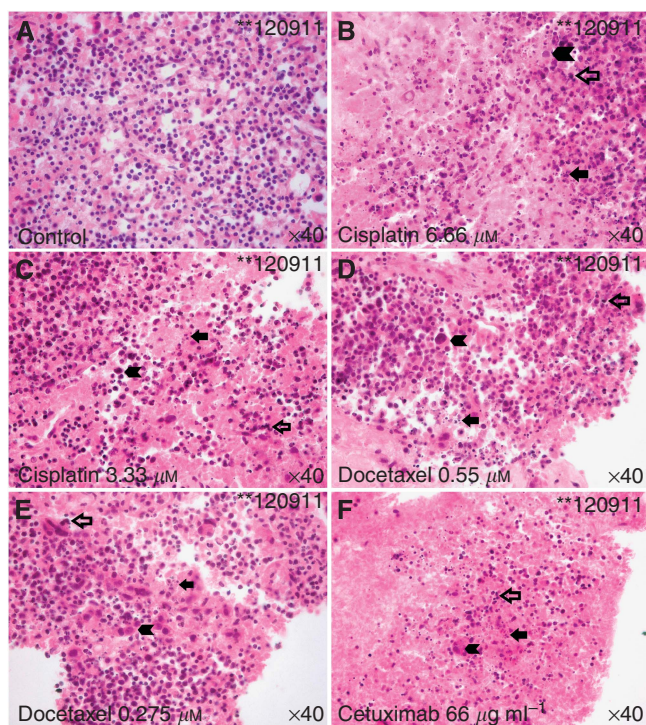
As the aim of this study was to evaluate effects of cytotoxic drugs in an attempt to establish a susceptibility assay, we restricted our further experiments to the period of 3–6 days *in vitro*.

An overview of the tissue samples used in this study and the experimental setup is given in Table 1.

**Treatment of HNSCC slices with cytotoxic drugs.** Slices were treated with cisplatin (3.33 and 6.66  $\mu\text{M}$ ; Desoize *et al*, 1996), cetuximab (66  $\mu\text{g ml}^{-1}$ ) and docetaxel (0.275 and 0.55  $\mu\text{M}$ ; Bisset *et al*, 1993) throughout the cultivation period of 3–6 days. Control slices of the same tumour were not exposed to cytotoxic drugs but cultured and fixed simultaneously. Potential cytotoxic effects of ethanol as diluent of docetaxel could not be detected, neither by HE-staining and count of nuclei (Figure 2Aa–c) nor by antibody staining against Ki-67 or caspase 3 (Figures 2Ad and 2Ae). All slices were then embedded in paraffin for histological analysis. In HE-stained sections of samples treated for 6 days, a higher number of fragmented nuclei was present compared with untreated controls; moreover, pycnotic alterations of nuclei, increased cellular polymorphisms and fragmented nuclei (Figures 3B–F) were prominent after cytotoxic treatment.

Next, we tested whether chemotherapy-induced changes could also be observed after reducing the period of treatment to 2 days. Slice cultures derived from a lymph node metastasis of a supraglottic squamous cell carcinoma were kept for 1 day without treatment and then exposed to chemotherapy for 2 days. Cell loss was evident in sections from all treated slices but not in controls (Figures 4B–G). Using the Image J plugin Cell Counter, we then quantified the numbers of nuclei. This analysis also revealed highly significant, dose-dependent cell loss induced by cytotoxic

Figure 2. Effect of cytotoxic drugs on cellular viability and intra-slice distribution of proliferation and apoptosis. (A) Effects of drug diluents on slices. (a) Number of nuclei in control and in slices tested with 0.08% ethanol as diluent of docetaxel (0.55  $\mu\text{M}$ ). (b, c) Morphology in HE-stained paraffin sections of slices cultured in solvent control (ethanol 0.08%) (c) does not visibly change compared with slices cultured in tumour medium only (b). (d, e) Staining for proliferation (Ki-67 (red)) and apoptosis markers (Caspase 3 (green)) does not provide any differences caused by the ethanol. (B) Vertical gradients in slices. (a–g) Investigation of transverse sections of the slices does not show any vertical gradient neither in proliferation (immunostaining for Ki-67 (red)) nor in apoptosis (immunostaining for caspase 3 (green)) after any of the tested treatment modalities. Arrows mark the upper and lower surface of each slice.



**Figure 3. Nuclear fragmentation after treatment.** (A) HE-stained untreated control kept *in vitro* for 6 days. Note the euchromatic nuclei and an absence of nuclear fragments. (B–F) Slices treated with cisplatin 6.66  $\mu\text{M}$  (B), cisplatin 3.33  $\mu\text{M}$  (C), docetaxel 0.55  $\mu\text{M}$  (D), docetaxel 0.275  $\mu\text{M}$  (E) or cetuximab 66  $\mu\text{g ml}^{-1}$  (F) exhibit fragmented nuclei (arrow, filled), pycnotic alterations (arrow, not filled) and cellular polymorphisms (arrow head) as hallmarks of apoptosis, which are most prominent after exposure to high dose of cisplatin (B) and cetuximab (F). Note that some regions seem to be depleted from cells.

treatment in one tumour (\*\*020712; Figure 4A). In another tumour (\*\*071111), the numbers of nuclei were not statistically different after 3 days of treatment, but determining caspase-3-positive cells revealed significant induction of apoptosis under treatment with docetaxel but not with other drugs (Figure 5). This provides an example of different susceptibility to cytotoxic drugs of individual tumours.

## DISCUSSION

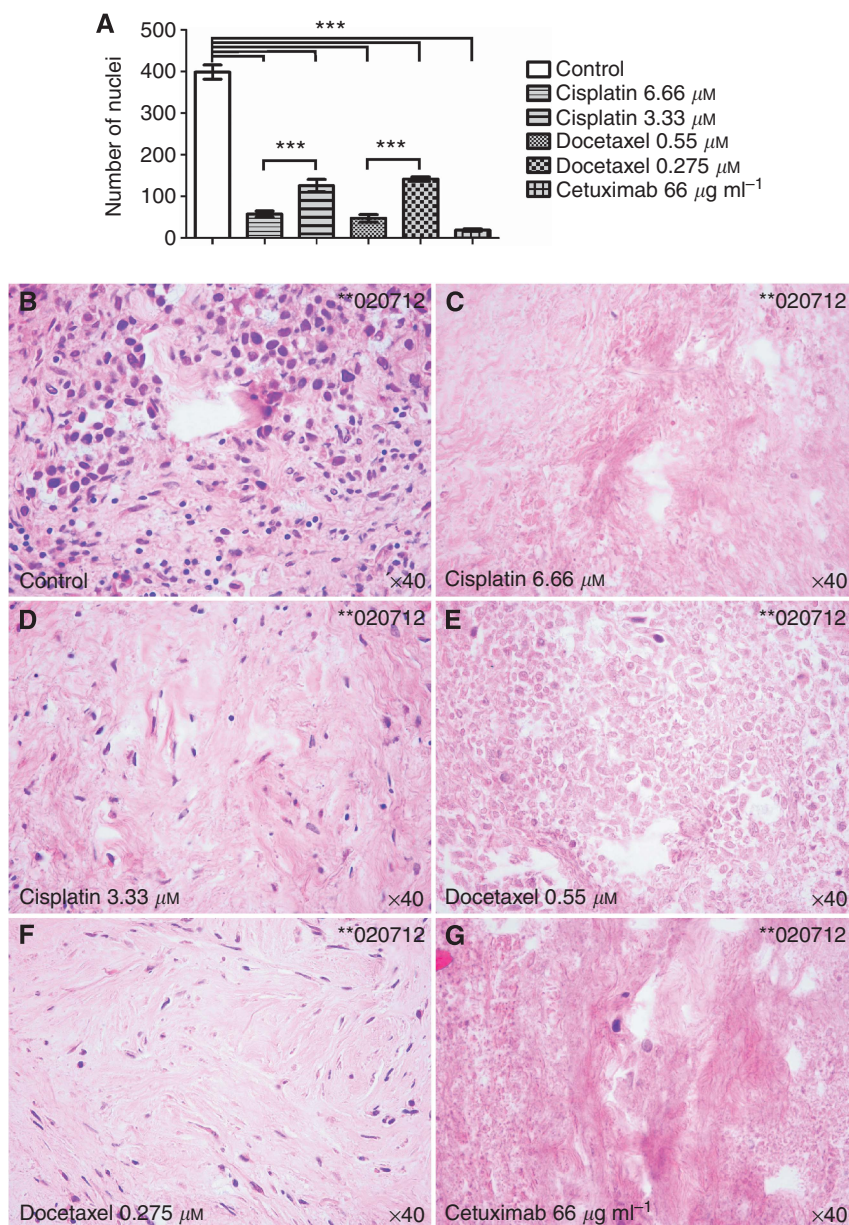
HNSCC is a heterogeneous group of tumours with highly relevant differences in their susceptibility to different cytotoxic drugs and radiotherapy. At present, it is difficult, if not impossible, to predict an individual's response to specific cytotoxic drugs, for example, based on biomarkers. Since the broad scale use of chemotherapy for treating human cancers, individual drug susceptibility has been studied using various test systems. Not surprisingly, the first studies have been performed in leukaemia, where access to cancer cells is undemanding (Bosanquet *et al*, 1983; Lihou and Smith, 1983; Park *et al*, 1983). For solid tumours, different test systems have been established, starting with clonogenic assays, which aimed at culturing isolated tumour cells in the presence of different cytotoxic drug concentrations *in vitro* in a manner much like bacterial antibiotic sensitivities. Contemporary studies work with human cancer xenografts transferred into mice, thereby creating a more true-to-life situation (Kamiyama *et al*, 2013). None of the technologies has yet gained significant clinical impact, which may

be due to the lack of translational research efforts from bedside to bench and back to bedside in most of the technologies. More importantly, a potential source of artifacts relies in the singularisation of cells using trypsinisation with the resulting isolation from their normal environment, which significantly impacts on tumour growth (Pietras and Ostman, 2010; Hanahan and Weinberg, 2011). For example, ECM epitopes provide important differentiation cues, for example, by signalling via Toll-like receptors (Schaefer *et al*, 2005; Senner *et al*, 2008; Moreth *et al*, 2010; Merline *et al*, 2011) and integrins (Abdollahi *et al*, 2005; Alghisi *et al*, 2009). Aiming at partial preservation of such signalling pathways, we previously used collagenase IV, which causes particulation rather than complete cellular isolation (Wichmann *et al*, 2009; Dietz *et al*, 2010; Wichmann and Dietz, 2011). Moreover, we used ECM proteins for coating of polystyrene surfaces allowing for specific interaction of integrins and other receptors with ECM, to further mimic *in vivo* conditions in the FLAVINO assay (Wichmann *et al*, 2009; Dietz *et al*, 2010, 2012; Horn *et al*, 2010; Schrader *et al*, 2012).

Here, we established organotypic slice cultures, which we have previously used to study mechanisms of neuroinflammation and neurodegeneration in rodent (Kluge *et al*, 1998; Hailer *et al*, 2001; Dehghani *et al*, 2003; Eyüpoglu *et al*, 2003; Proding *et al*, 2011) and human neural tissues (Nitsch *et al*, 2000; Merz *et al*, 2013). In the latter (deriving from epilepsy surgery), we have shown that human tumour-necrosis-factor-related-apoptosis-inducing ligand, which is not toxic in mice (Walczak *et al*, 1999), induced lysis of non-transformed brain cells (Nitsch *et al*, 2000). Such highly relevant species differences gained world-wide attention after the 'London tragedy' in 2006, when six volunteers developed toxic shocks and cytokine storms after administration of TGN1412, an anti-CD28 antibody, which was tested before in animals (Kenter and Cohen, 2006; Dowsing and Kendall, 2007; Merz and Bechmann, 2011; Seok *et al*, 2013), further arguing for the development of human test systems for drug toxicity. The London incidence may explain the current renaissance of this idea (van der Kuip *et al*, 2006; Merz *et al*, 2010; Vaira *et al*, 2010; Merz and Bechmann, 2011) despite the ASCO's decision to abolish chemosensitivity testing in 2004 due to a lack of reliability or clinical practicability of available assays at that time (Schinköthe *et al*, 2007). This is where we see a potential advantage of the human tissue slice culture technology: they can quickly be set up while the complex cross-talk between matrix and cells as well as the individual cellular composition and susceptibility to drugs is preserved. In addition, healthy surrounding tissues such as stratified muscle cells and glands also exhibited excellent preservation (Figures 1Af and 1Ag). Vivid proliferation was still visible after 6 days *in vitro* using Ki-67 staining (Figures 1B, 2A and 2B). In untreated slices, it was possible to distinguish between the different components of the tissues and to evaluate the composition of the slice, even if the slices are certainly heterogeneous.

Following treatment with cytotoxic drugs, nuclear fragmentation was abundantly present in HE sections, and apoptosis induction was confirmed using antibodies to activated caspase 3 (Figures 3 and 5). Quantification of nuclei revealed significant cell loss after treatment (Figure 4). Whereas we could show late effects of the tested drugs by the visualisation of lower cell density through cell loss, earlier stages of apoptosis could be quantified by the cleavage of caspase 3. As the individual tumours react at different rates, it is important to consider both early and late stages of cell death, which is why we quantified both features in the samples.

We could show that activated caspase 3 is spread evenly throughout the slice rather than showing predominant appearance at the surfaces (Figure 2B) proving that drug penetration into the slices takes place. As for potential side effects of the drugs used here, we cannot make judgment on systemic effects, but we can



**Figure 4.** Cell loss after treatment. **(A)** Number of nuclei before and after treatment with cisplatin, docetaxel or cetuximab. After treatment for 2 days, cell loss was significant in all treated samples compared with untreated controls of the same tumour. Statistics were performed using GraphPad Prism 5 and one-way ANOVA, and  $P < 0.05$  was considered significant. High doses of cisplatin and docetaxel resulted in significant higher amount of cell loss compared with lower concentrations tested. **(B–G)** HE-stained paraffin sections of slices treated with various cytotoxic drugs: cisplatin 6.66  $\mu\text{M}$  **(C)**, cisplatin 3.33  $\mu\text{M}$  **(D)**, docetaxel 0.55  $\mu\text{M}$  **(E)**, docetaxel 0.275  $\mu\text{M}$  **(F)** and cetuximab 66  $\mu\text{g ml}^{-1}$  **(G)**. \*\*\* $P < 0.0001$ .

state that damage to the surrounding tissue would be visible in our experiment if it was consistently caused by an individual drug. As systematical follow-up of every aspect in each of the tumours tested was not possible due to the limited sample volume not needed for proper diagnosis, different aspects (e.g., viability over time or reaction to chemotherapeutics) therefore had to be addressed with different tumours.

The next step will be to test in a prospective study whether and which parameters of susceptibility to treatments of an individual tumour correspond to the respective patient's response and clinical outcome. Only if this is the case, cultivation conditions can be regarded as mimicking the *in vivo* situation faithfully.

The combined analysis of cell death (caspase 3 and cell density) is certainly time consuming and relies on an experienced, un-biased

investigator. Instead of counting cells by hand or measuring cell density, we believe that measurement of biochemical markers in the supernatant or in homogenates of the slices will provide a more feasible readout of such an assay. Histology performed in our study was primarily designed to establish the tissue quality and longevity as well as detecting the effects of drugs in principle. For the general use in laboratory routine, the readout should be made as standardised and least time consuming as possible. Furthermore, kinetics are not uniform in all tumours, and care must be taken not to oversee drug effects when using cell density as paradigm. Therefore, we are testing alternate approaches to quantify cell death, for example, using soluble markers (LDH, M30, CK18) present in the TM, which may also allow for monitoring treatment-induced degeneration over time, or tissue homogenates for the analysis of RNA and/or proteins.

Table 1. Detailed information about tissue samples used in this study

Sample	Tissue type/origin	Duration of cultivation		Slices acquired with	Fixation time
**200111	Carcinoma of the larynx	5 h, 6d	Method establishment	Vibratome	None (tissue not cultivated)
**190411	Carcinoma of the trachea	1d, 2d, 6d, 9d, 12d	Method establishment	Vibratome	24 h
**270611	Carcinoma of the tonsil	6d	See data (Figures 1A and 1B)	Vibratome	24 h
**080611	Metastasis of an oropharyngeal carcinoma	5d, 7d	See data (Figure 1B)	Vibratome	48 h
**120911	Metastasis of a carcinoma of the tonsil	6d	See data (Figure 3)	Tissue chopper	18 h
**071111	Carcinoma of the base of tongue	3d, 6d	See data (Figures 1C and 5)	Tissue chopper	24 h
**070512	Carcinoma of the larynx	4d	Method/analysis establishment	Tissue chopper	24–48 h
**160512	Carcinoma of the base of tongue	4d	Method/analysis establishment	Tissue chopper	24–48 h
**240512	Carcinoma of the base of tongue	4d	Method/analysis establishment	Tissue chopper	24–48 h
**020712	Metastasis of a supraglottic carcinoma	3d, 5d	See data (Figure 4)	Tissue chopper	24–48 h
**040713	Carcinoma of the pharynx	3d, 5d	See data (Figures 1A and 1C)	Tissue chopper	24 h
**040713	Metastasis of a carcinoma of the pharynx	3d, 5d	See data (Figures 1A, 2A and 2B)	Tissue chopper	24 h

This table presents the type of the tumour tissues used in this study, the duration of cultivation, the cutting method, the use of each tumour and where the data are shown as well as the fixation time. The approach in our study was the following: We first tested vibratome sections (samples \*\*200111, \*\*190411, \*\*270611 and \*\*080611) of which only three could be kept in culture with good preservation until day 6 (\*\*200111, \*\*270611, \*\*080611). As we found evident deterioration at day 7 in sample \*\*080611, we did not focus any longer on establishing a maximal survival time for individual tumours. Our reasoning was that for the purpose of the method, it is much more important to establish a minimum survival time suitable to test drug effects. We found that slices cut with a tissue chopper were faster to obtain and more uniform in thickness and exhibited very good tissue preservation until day 6. Therefore, we looked at the effects of treatments on day 4 (samples \*\*070512, \*\*160512 and \*\*240512) or – if there was enough tissue – on days 3 and 5 (samples \*\*020712, \*\*040713TM and \*\*040713Met).

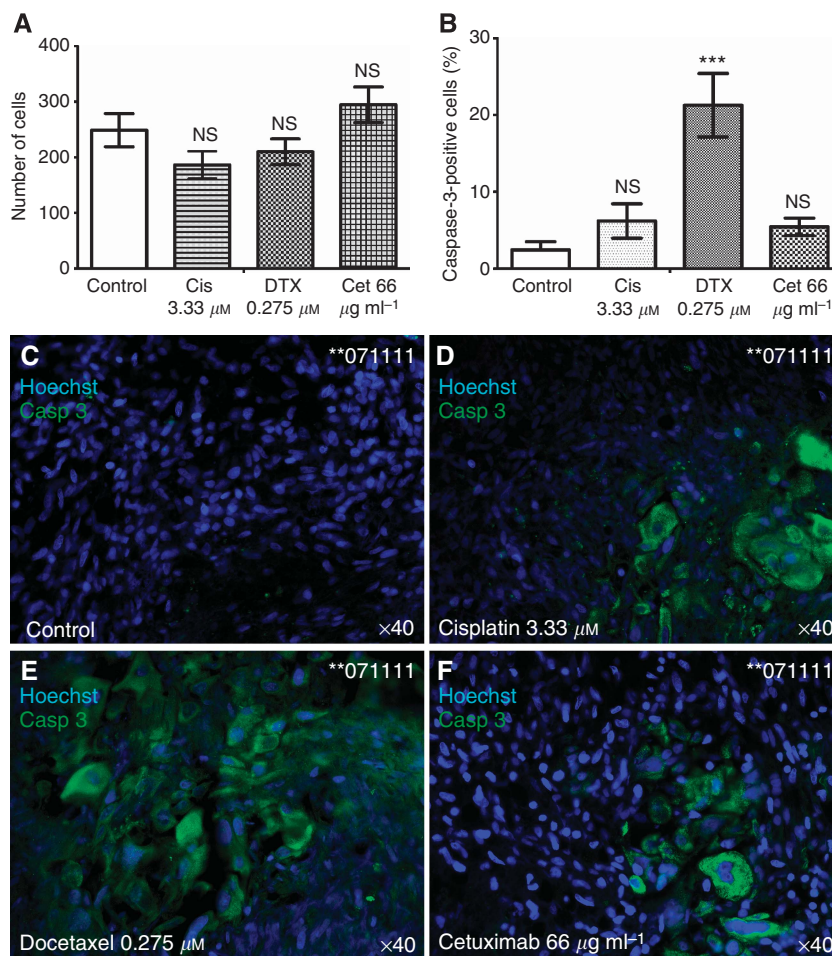


Figure 5. Activation of caspase 3 after treatment. (A) The number of Hoechst-positive nuclei in paraffin sections of a carcinoma of the base of the tongue did not reveal significant effects of treatments. (B–F) However, in sections co-stained with antibodies against activated caspase 3 (green), increased apoptosis was evident in all the treated samples compared with controls, but only docetaxel (DTX) treatment was considered significant (21.3% relating to total number of Hoechst-stained nuclei vs 2.5% in control). After treatment with cisplatin, 6.2% of all nuclei showed activated caspase 3, and after treatment with cetuximab, 5.4% of the cells were positive. \*\*\**P*<0.0001.



However, our current data demonstrate that HNSCC-derived slices can be kept in culture, may serve as prediction assay and can be used to better understand the mechanisms of tumour resistance by harvesting surviving tumour cells after treatment. Decision making between highly crucial different treatment options such as mutilating laryngectomy or organ-preserving primary chemoradiation (with potentially severe late complications) would highly benefit from a reliable predictive test system.

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