3D cell culture alters signal transduction and drug response in head and neck squamous cell carcinoma

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Abstract. Epidermal growth factor receptor (EGFR) upregulation is a typical characteristic of head and neck squamous cell carcinoma (HNSCC). However, tyrosine kinase inhibitors have not yet been able to achieve enough therapeutic benefit in clinical trials to justify their use in standard therapy regimens. At present, little is known about the reasons for this treatment failure. In the present study, the HNSCC cell lines UM-SCC-11B and UM-SCC-22B were tested for their response to tyrosine kinase inhibitors (TKI) under 2D and 3D cell culture conditions. Absorption and luciferase-based viability assays were used for this, as well as optical evaluation via fluorescence microscopy. In addition, EGFR and HER3 expression as well as the downstream signalling pathways PI3K/AKT/mTOR and RAS/RAF/MEK/ERK were investigated using western blotting. Cell line UM-SCC-11B revealed a strong resistance to lapatinib under 3D cell culture conditions, while a good response to TKI therapy was observed under 2D cell culture conditions. An associated overexpression of phosphorylated HER3 under 3D cell culture conditions offered a plausible explanation for the altered treatment response. The results of the present study represent an idea of how signalling mechanisms of cancer cells can be changed using different cell culture methods. Overall, 3D cell culture could be an important component in the analysis of resistance mechanisms in cancer therapy.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide and by far the most common malignancy of the upper aerodigestive tract (1). The main risk factors are alcohol and tobacco abuse which act synergistically (2,3). Despite the development of new treatment strategies in the last decades, the median five-year survival rate for HNSCC remains around 50%. Since, in about 90% of cases, the tumour overexpresses the epidermal growth factor receptor (EGFR), it has become a region of interest for novel therapy approaches (4).

EGFR, also known as HER1, together with HER2, HER3 and HER4, belongs to the group of receptor tyrosine kinases (RTK). Receptor activation is initiated by ligand binding. This leads to an autophosphorylation of the RTK via homodimerization with a similar receptor or heterodimerization with other RTKs. HER3 is unique in that it does not have sufficient autophosphorylation ability. Therefore, it must be activated by heterodimerization with other RTKs. In downstream signal transduction, the PI3K/AKT/mTOR and the RAS/RAF/MEK/ERK pathways are of particular relevance. Each RTK can be inhibited in its phosphorylation by tyrosine kinase inhibitors (TKI) (5). In this study, we used lapatinib and afatinib. Lapatinib only inhibits HER1 and HER2, while afatinib inhibits each of the four receptors HER 1-4.

A well-known example of successful TKI treatment is chronic myeloid leukaemia (CML). Imatinib was the first TKI against BCR-ABL fusion protein, and it became known as a breakthrough in targeted therapy (6). TKIs were then tried in many tumour entities with varying degrees of success. Afatinib is approved for the treatment of locally advanced or metastatic NSLC and lapatinib for the treatment of HER2-positive breast cancer (7,8).

RTK-directed therapies have hardly any clinical relevance in the therapy of HNSCC, except for the monoclonal EGFR antibody cetuximab (9). Presently, there is insufficient evidence supporting the use of TKI, such as lapatinib or afatinib, in the therapy of HNSCC. Therefore, they are in most cases used for clinical trials (10,11).

Preclinical studies have already produced promising results for some TKIs, including lapatinib and afatinib; however, in clinical studies, lapatinib has shown no additional benefit compared to established therapy concepts (12,13). Neither have mono kinase inhibitors such as Erlotinib or Gefitinib been able to confirm the preclinical results (14), while more promising results were obtained for afatinib. A

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phase 3 trial identified a subpopulation of patients (p16-negative, EGFR-amplified, HER3-low, PTEN-high) who could benefit from afatinib treatment (15). The identification of such subpopulations and the investigation of resistance mechanisms is essential for the application of targeted therapies. Various resistance mechanisms are discussed as reasons for the poor response of TKIs in HNSCC. Low mutation rates of RTKs in HNSCC must be considered as well as receptor-independent activation of downstream signalling pathways (14,16).

3D cell culture is becoming increasingly popular in examining resistance mechanisms. The idea is to create a better imitation of the *in vivo*-conditions through more cell-cell interactions and different growth behaviours. There are already studies showing a correlation between treatment response under 3D cell culture and clinical outcome (17). Furthermore, 3D cell culture can be set up with hypoxic areas as well as coculture with stromal cells (18).

The HNSCC is supposed to include the presence of cancer stem cells (CSC), which have increased drug resistance and are capable of regenerating tumour tissue after chemotherapy. Prince *et al* were the first to detect $CD44^{+}$ cells as a population with stem cell-typical properties in HNSCC (19). Since then, there has been increasing interest in defining this subpopulation of cells. More additional markers, such as ALDH1A1, have been found, but an exact definition of CSC has yet to be determined (20,21), and 3D cell culture as a link between conventional 2D culture and xenograft studies could be a key factor here (22).

There is evidence that 3D cell culture can change the response and signal transduction of tumour cell lines (23-25). We therefore investigated the response behaviour of TKIs in HNSCC using 3D cell culture. Our focus was to compare 2D and 3D cell cultures in terms of their response behaviour to lapatinib and afatinib. Additionally, we examined the underlying signal transduction.

Materials and methods

Cell culture and drug treatment. The cell lines UM-SCC-11B (11B), UM-SCC-22B (22B) and UM-SCC-74A (74A) were obtained from Dr T.E. Carey (University of Michigan, Ann Arbor, MI, USA). The cell line UD-SCC-1 was obtained from Professor Wagenmann (University of Düsseldorf, Germany). These lines originated from squamous cell carcinomas (SCC) of the larynx (11B), the hypopharynx (22B), the tongue (74) and the oropharynx (UD01). STR profiling was performed to test the authenticity of the cell lines. The cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific, Inc.) supplemented with 10% foetal calf serum (FCS) and antibiotics (Gibco, Life Technologies). 11B and 22B were chosen for 3D cell culture for their ability to form stable and viable spheroids when cultivated in low attachment plates.

For 3D cell culture, the cells were suspended at a density of 2.5×10^{5} cells/ml and transferred into 96-well Nunclon Sphera-Treated U-shaped-bottom Microplates (Thermo Fisher Scientific, Inc.), so every spheroid contained 50.000 cells at the beginning of cultivation. Spheroids were incubated for seven days prior to treatment, with fresh mediums replaced every 48 h.

For drug treatment, we used afatinib and lapatinib (Selleck Chemicals) diluted in dimethyl sulfoxide (DMSO) and corresponding DSMO concentrations as the negative control. These were added to the cells in 2D or 3D cultures with concentrations ranging from 1 to 50 μM, depending on the experiment. The cells were incubated with the TKIs for 48 h.

Alamar blue viability assay. Viability testing on 2D cell culture was performed via Alamar Blue viability assay (Thermo Fisher Scientific, Inc.). After treatment with TKI, 10 μl Alamar Blue solution was added to each well, according to the manufacturer's instructions. Absorption was measured after 3 h using an Infinite M Plex Microplate Reader (Tecan Trading AG).

3D viability assay. The spheroids were transferred in 96 well white clear bottom plates (Thermo Fisher Scientific, Inc.) and lysed with 100 μl CellTiter-Glo viability assay solution, according to the manufacturer's instructions. Luminescence was measured after 30 min using an Infinite M Plex Microplate Reader (Tecan Trading AG).

Western blotting. Cells were washed with PBS and lysed with RIPA Buffer (Sigma-Aldrich; Merck KGaA). Protein concentration of the cell lysates was calculated by DC Protein assay (Bio-Rad Laboratories Inc.) according to the manufacturer's instructions, and each probe was diluted to samples of 10 μg of protein. Samples were mixed with 3.75 μl loading dye and separated by SDS-page. The separated proteins were transferred to a polyvinylidene fluoride membrane and then blocked with a TBST buffer and 5% non-fat dry milk. Incubation with primary antibodies took place overnight. The primary antibodies used were EGFR, pEGFR, HER3, pHER3, AKT, pAKT, ERK, pERK and GAPDH. After three washing steps with the TBST buffer, the membrane was incubated with a secondary antibody followed by three additional washing steps. For luminescence detection, the membrane was coated with 1 ml luminol and 1 ml peroxide solution and then analysed with an IBright FL 1000 (Invitrogen; Thermo Fisher Scientific, Inc.).

Microscopy. For visual analysis of viability in 3D culture, cells were dual-stained with ATP-Red dye (Sigma-Aldrich; Merck KGaA) as a marker for viable cells and Sytox-Green dye (Thermo Fisher Scientific, Inc.) as a marker for apoptotic/necrotic cells. For the staining of cells under 2D culture, we used Calcein AM as a marker for viable cells and Ethidiumhomodimer-1 for apoptotic/necrotic cells (Thermo Fisher Scientific, Inc.). After staining, the cells were cultured in 2D or 3D conditions and treated with lapatinib or afatinib, as previously described. Pictures were taken via light microscopy (Axiophot, Fa. Zeiss).

Statistical analysis. Each experiment was repeated three times (n=3) with duplicates, and mean values as well as the standard deviations were calculated. Viability was calculated in comparison to the corresponding control group. IC_{50}-doses were calculated from the resulting dose-response curves

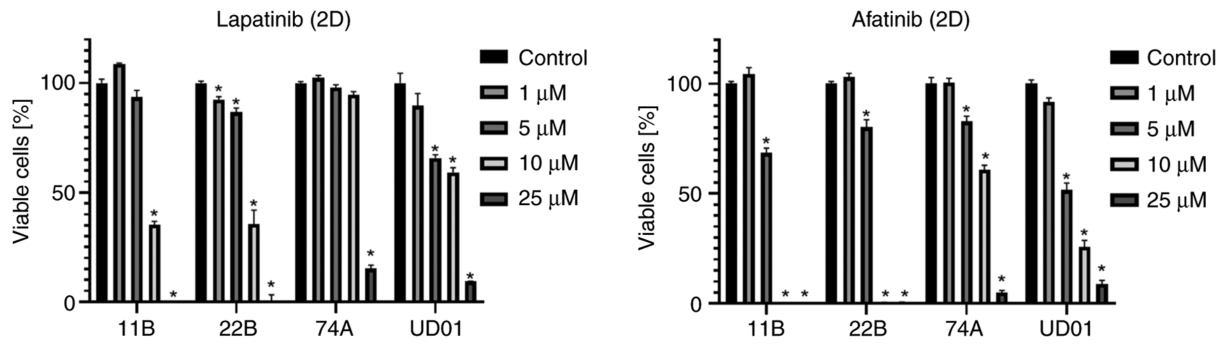

Figure 1. Viability analysis of 11B, 22B, 74A and UD01 cells after treatment with TKI for 48 h. Viability was calculated in comparison to the corresponding control group. Empty bars resemble that no viable cells could be detected. The data are presented as mean values ± SD (n=3), *P≤0.05 vs. control.

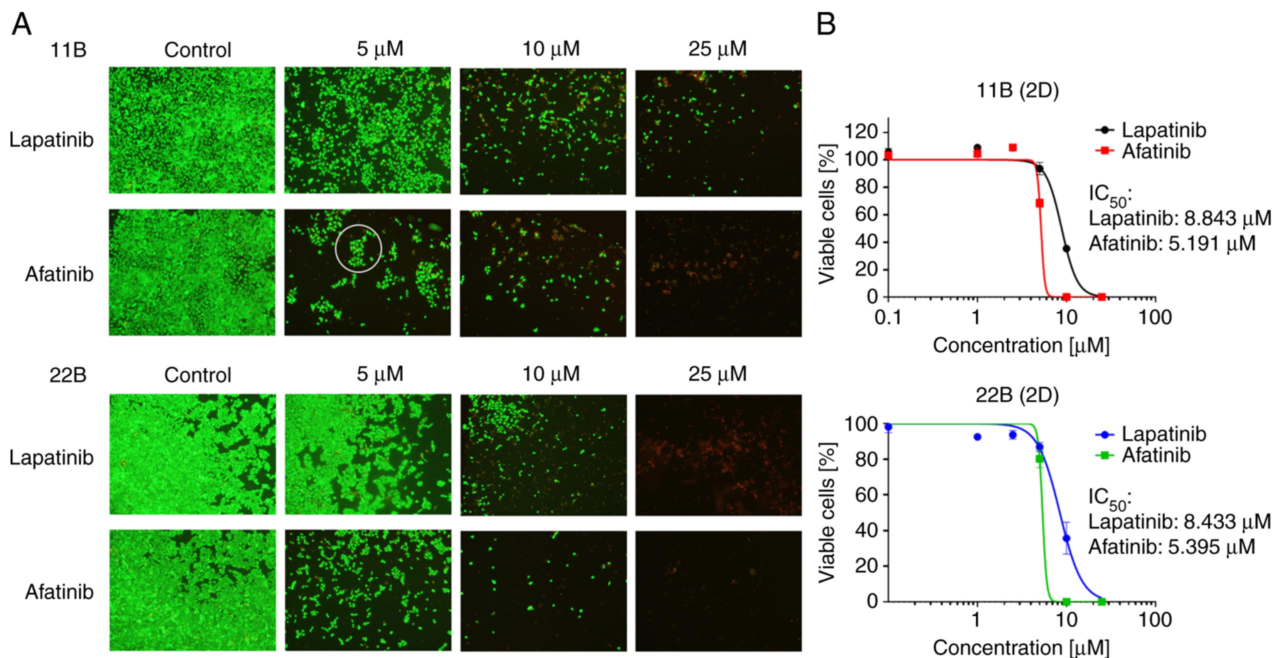

Figure 2. (A) Fluorescence Microscopy of 11B and 22B cells in 2D cultures, stained with Calcein AM/Ethidiumhomodimer-1, after treatment with TKI for 48 h (magnification, x100). Living cells are shown in green; red cells resemble apoptotic/necrotic cells. The white circle highlights an example of the colony formation of 11B cells. (B) Dose/response curves for 11B and 22B cells in 2D culture under treatment with TKI for 48 h. The data are presented as mean values ± SD, n=3.

using Graph Pad Prism. Resistance was considered as an IC_{50}-dose >50 μM. Differences between multiple groups were analysed using one-way ANOVA followed by Tukey's post hoc test. A probability value of P≤0.05 was considered significant.

Results

HNSCC cell lines have a high response to TKI therapy in 2D culture. All the used cell lines responded very well to TKI therapy in 2D cell cultures in terms of viability. Treatment with 5 μM afatinib resulted in a significant decrease of viability in each of the four cell lines. Under treatment with 25 μM lapatinib or afatinib, little-to-no viable cells could be detected (Fig. 1). The evaluation of IC_{50} values of 11B and 22B resulted in 8.843 μM (11B) and 8.433 μM (22B) for lapatinib. The IC_{50} values for afatinib were 5.191 μM (11B) and 5.395 μM (22B). In addition, the cells were photographed under viability staining and treatment with TKI. Concentrations were chosen close to their IC_{50} values (Fig. 2). With increasing concentration, the cell density and viability decreased. Under treatment with 5 μM afatinib, 11B cells formed up clusters. In areas with viable cell colonies, those cells seemed to be attached to each other.

3D cell culture alters treatment response to lapatinib in 11B cells. In 3D cell culture, we were able to determine a strong resistance of 11B to lapatinib (IC_{50}>50 μM). However, there was a strong response to afatinib (IC_{50}=7.822 μM) comparable to the IC_{50} seen in 2D culture. Cell line 22B showed no resistance to lapatinib (IC_{50}=9.666 μM) or afatinib (IC_{50}=2.2362 μM) in 3D cell culture either. These results were re-evaluated using microscopy (Fig. 3). The lapatinib-resistant cell line 11B showed a homogeneous distribution pattern of viable cells in the spheroid. Even with 25 μM lapatinib, there was hardly any difference

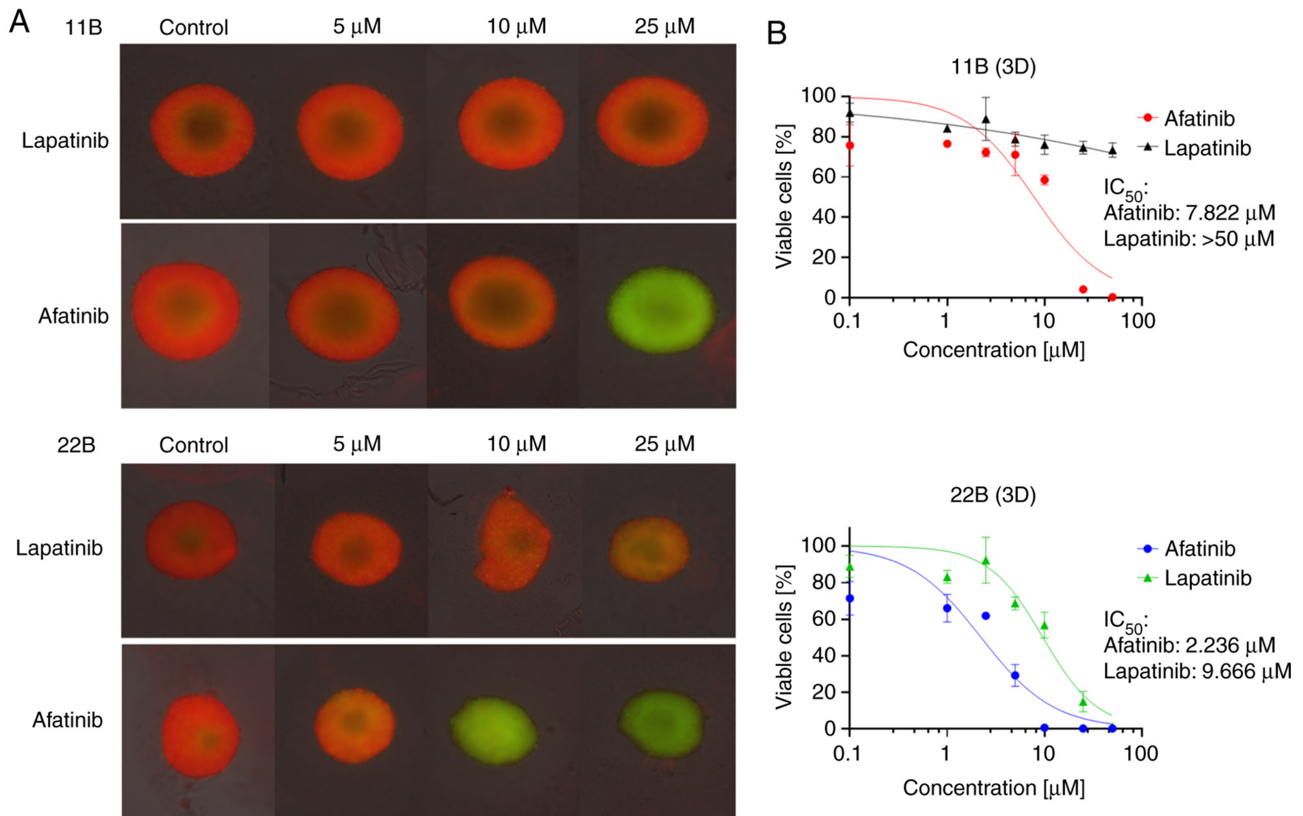

Figure 3. (A) Fluorescence microscopy of 11B and 22B cells in 3D cultures, stained with Sytox-Green/ATP-Red, under treatment with TKI for 48 h (magnification, x100). Living cells are shown in red; green cells resemble apoptotic/necrotic cells. (B) Dose/response curves for 11B and 22B cells in 3D culture under treatment with TKI for 48 h. The data are presented as mean values ± SD, n=3.

compared to the control. A very homogeneous distribution pattern of apoptotic/necrotic cells with hardly viable cells was found in spheroids of both cell lines when treated with 25 μM afatinib.

11B shows higher phosphorylation in 3D cell culture. Due to the limited response of 11B to lapatinib that was explicitly present in the 3D culture, both cultures were analysed via western blot after treatment with lapatinib (Fig. 4). A comparison of the two culture models in the western blot showed a constantly high EGFR expression in both 2D and 3D cultures. In 3D cell culture, the phosphorylation of EGFR was stronger. This was also observed with HER3 and the downstream signalling proteins AKT and ERK. The phosphorylation of EGFR decreased after treatment with lapatinib, whereas the phosphorylation of HER3 remained consistently high independent of the TKI concentration.

Resistant cell line 11B has higher expression of HER3. To compare the two cell lines regarding their different behaviour in 3D culture, a western blot analysis of cell lines 11B and 22B treated with afatinib and lapatinib was carried out in 3D culture (Fig. 4). Both lines showed similar expressions of EGFR and a reduction in EGFR phosphorylation under treatment with afatinib or lapatinib. As previously mentioned, a constantly high HER3 phosphorylation could be detected in line 11B in 3D culture. This phosphorylation was suppressed under treatment with afatinib. In line 22B, hardly any pHER3 expression was detectable even under control conditions.

A similar result was observed with the downstream signalling protein AKT. Neither lapatinib nor afatinib influenced phosphorylation in line 11B. In contrast, pAKT was hardly detectable for line 22B. A strong ERK expression was detected in both cell lines. Lapatinib treatment had no effect on either the expression of panERK nor of pERK. On the other hand, afatinib treatment showed a slight decrease in ERK phosphorylation in 11B and a complete blockage of phosphorylation in line 22B.

Discussion

We aim to use 3D cell culture for generating a deeper understanding of cell-cell interactions and resistance behaviour of tumour cells. The 3D cell culture appears to be more suitable for this issue than the conventional 2D cell culture for several reasons: a changed growth rate, better imitation of hypoxic areas, and a changed drug response (26-28). There are several publications on comparisons of drug response as well as protein and gene expression between 2D and 3D cell cultures (29,30). In the case of HNSCC, hardly any studies on the response to TKI in 3D have been performed. We therefore addressed the question of why, despite promising preclinical results, there has so far been little clinical success with TKI in the treatment of HNSCC. In the present work, we were able to observe an altered treatment response to TKI using a 3D cell culture model and to provide explanatory approaches for this phenomenon.

Cell-cell interaction is essential for survival. Preliminary tests have shown that all our HNSCC cell lines show a very

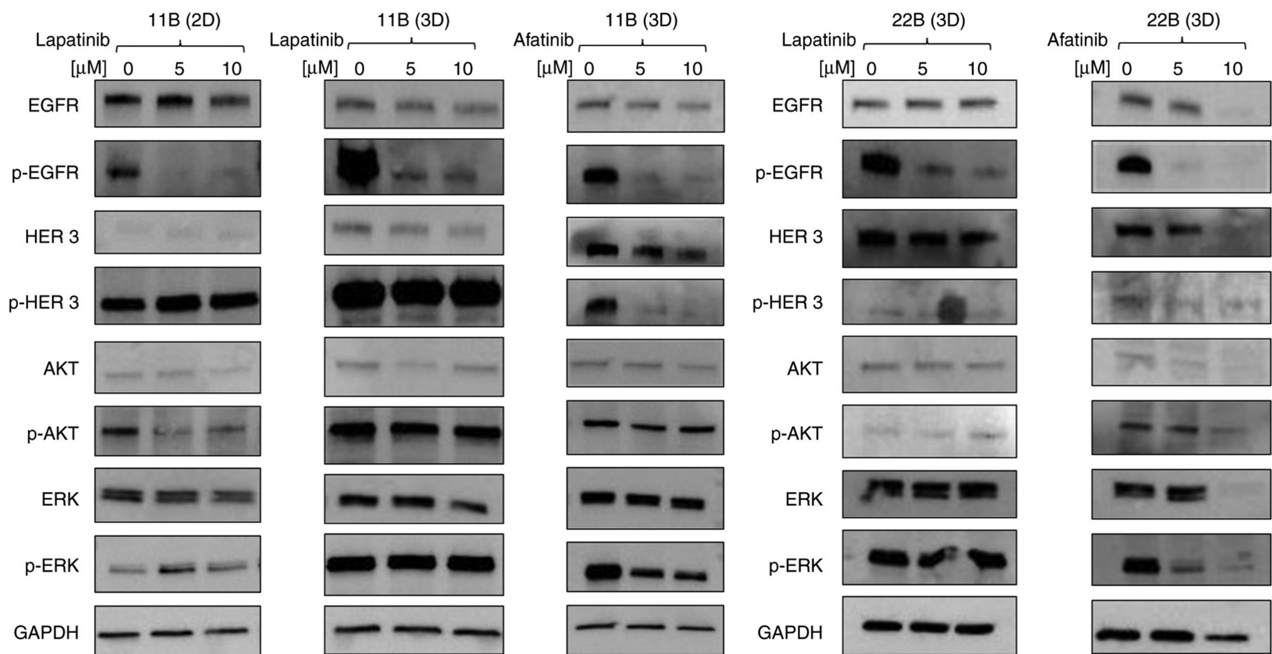

Figure 4. Western blotting analysis of cell lysates acquired from 11B and 22B cells in 2D or 3D cultures after treatment with lapatinib or afatinib for 48 h. EGFR, epidermal growth factor receptor; p-, phosphorylated.

similar and strong response to TKI in 2D cell culture. We therefore selected two cell lines that were reliably capable of spheroid formation to examine them more closely in 3D. In the microscopic examination of the 2D cells, we observed a colony formation of the 11B cells, especially under treatment with 5 μM afatinib. This seems to represent a survival benefit for the cell when it is in contact with other cells. The reason for this could be altered signal transduction via growth factors, which would also be in line with the higher IC_{50} doses that tend to be observed in 3D cell cultures. In our experiments, we observed some sort of resistance in cell line 11B, with an IC_{50} dose >50 μM for lapatinib. It seems that the cells have also changed their characteristics because of the changed growth conditions. Even if a wide variety of mechanisms are possible for this, it was likely that changes in the EGFR cascade have occurred. This presumption was confirmed in the western blot analysis. Both EGFR and HER3, as well as their downstream signal molecules AKT and ERK, had higher phosphorylation under 3D cell culture than under conventional 2D cell culture.

HER3 as a possible resistance inductor. The observed strong phosphorylation of the HER3 receptor in cell line 11B offers a plausible explanation for the observed resistance to lapatinib, while maintaining its sensitivity to afatinib. HER3 is already a discussed option for overcoming resistance to EGFR-directed therapy in other tumour entities (31,32). For HNSCC, there are preclinical approaches to circumvent cetuximab resistance using HER3 targeting (33). A frequently observed problem of cetuximab therapy is acquired resistance, which describes a therapy failure that occurs only after some time under medication. It is assumed that an upregulation of other tyrosine kinases of the EGFR family contributes to this behaviour. Therefore, it seems that a therapy strategy aimed at the entire EGFR family, as is already being used in NSCLC, seems promising (34). We therefore assume that successful clinical usage of TKI depends on how many opportunities there are left for the development of resistance. From a preclinical point of view, this explains why afatinib could be superior to therapy with lapatinib, erlotinib or gefitinib.

The data from clinical studies on TKIs do not yet justify their use in HNSCC (35). Afatinib is still the most promising substance among the TKIs. A phase 3 study by Guo *et al* evaluated the progression-free survival of patients undergoing second-line therapy for HNSCC. They detected significantly longer progression-free survival for afatinib in comparison to methotrexate (36). In this regard, the results of our study are in line with the clinical data.

A limiting factor in our study is certainly the small number of lines examined. Therefore, even more cell lines or, ideally, patient-derived spheroids would have to be examined to confirm that the increased activation of pHER3 is a potential resistance mechanism in 3D cell culture. However, the increased phosphorylation activity observed in our cell line is a good example of the changed signal transduction processes under different culture conditions.

Altered conditions result in altered reactions. The present study aims to address the leading question, why tumour cells tend to have a limited treatment response under 3D cell culture. One possibility would be that the increased contact between the cells and the overall higher cell density would also lead to increased communication between the cells. We were also able to observe that the concentration of receptors and intracellular signalling molecules of the EGFR family tends to be higher in 3D cell cultures than in normal 2D cell cultures. Whether this occurs through increased cell-to-cell communication or through intracellular mechanisms would have to be investigated more closely.

Another explanation for a higher rate of surviving cells in the spheroids is based on the life cycle of the cancer cells. Since mitosis is a very energy-consuming process, dormant cells can

often be found in hypoxic and nutrient-undersupplied areas. These cells are less susceptible to cytostatic treatment and therefore show higher resistance behaviour (37). In the 3D cell culture model, there are significantly more dormant cells due to the different nutrient and oxygen content in the spheroid.

The model of the cancer stem cell niche assumes that such resistant cells are arranged in sub-areas. This hypothesis is particularly widespread in HNSCC research. Compared to regular tumour cells, tumour stem cells have an increased potential for regeneration and metastasis. However, this stem cell potential seems to be dependent on the environment of the stem cell, including supportive tumour cells and tumour stromal cells (38). It remains to be seen whether these stem cells occur more frequently in 3D cell cultures.

The most likely cause of changed resistance behaviour in 3D cell cultures is a combination of various effects. Therefore, it is even more important to establish a 3D cell culture that gives a good representation of the *in-vivo* conditions. Our long-term goal is to develop a 3D cell culture using bioprinting that includes patient-derived cells and supportive cells (39). An optimized 3D cell culture model would then provide the basis for further resistance testing of various drug groups. Possible new resistances that arise from the different culture techniques could be examined by means of genetic analysis. One possibility would be the comparative RNA sequencing analysis between 2D and 3D culture or between different forms of 3D culture.

Even though 3D cell culture is associated with longer cultivation times and higher material costs, we believe that these costs are reasonable if the method is well established and used efficiently.

In conclusion, we found a HNSCC cell line (11B) with an altered treatment response to lapatinib under 3D cell culture. In addition, we found an upregulation of HER3 phosphorylation, which is a plausible explanation for a resistance mechanism. Limited therapy response compared to conventional cell culture is a frequently observed phenomenon in clinical trials. We see this as an indication that the 3D cell culture could be superior to the conventional 2D cell culture for the investigation of drug resistance development in cancer therapy.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JH wrote the initial manuscript. AL and JK conceived and designed the present study. Experiments and data collection were completed by JH, YJ and ET. AA and NR interpreted the results and helped with the writing of the manuscript. JH and JK confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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