

Evaluation of spheroid head and neck squamous cell carcinoma cell models in comparison to monolayer cultures

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Received February 16, 2015; Accepted April 24, 2015

DOI: 10.3892/ol.2015.3487

Abstract. Two-dimensional (2D) monolayer cell culture models are the most common method used to investigate tumor cells *in vitro*. In the few last decades, a multicellular spheroid model has gained attention due to its adjacency to tumors *in vivo*. The aim of the present study was to investigate immunohistochemical differences between these two cell culture systems. The FaDu, CAL27 and SCC25 head and neck squamous cell carcinoma (HNSCC) cell lines were seeded out in monolayer and multicellular spheroids. The FaDu and SCC25 cells were treated with increasing doses of cisplatin and irradiation. CAL27 cells were not used in the proliferation experiments, since the spheroids of CAL27 cells were not able to process the reagent in CCK-8 assays. Furthermore, they were stained to present alterations of the following antigens: Ki-67, vascular endothelial growth factor receptor, epithelial growth factor and survivin. Differences in growth rates and expression patterns were detected in certain HNSCC cell lines. The proliferation rates showed a significant divergence of cells grown in the three-dimensional model compared with cells grown in the 2D model. Overall, multicellular spheroids are a promising method to reproduce the immunohistochemical aspects and characteristics of tumor cells, and may show different response rates to therapeutic options.

Introduction

Malignant growth in the head and neck area affects a large group of individuals and remains a serious health issue; worldwide, >600,000 new cases of head and neck squamous cell carcinoma (HNSCC) are diagnosed every year (1). Within this group, cancer of the lips and the oral cavity are

the most frequent, with an incidence of nearly 250,000 cases annually (1). The five-year relative survival rate varies from 20-90% depending upon the primary tumor site of the tumor and the clinical stage at the presentation of disease (2).

Treatment options for HNSCC include surgery and/or radio(chemo)therapy. Despite the plethora of novel chemotherapeutic agents, refined radiation protocols and carefully selected patients for surgery, the overall survival of affected patients has only improved marginally during the last 30 years (3). Nevertheless, the requirement for novel therapeutic approaches remains undisputed.

One common approach for the identification of potential therapeutic agents is tests in a monolayer cell culture system (4). In the last 50 years, a multitude of oncological studies have been performed with this classic two-dimensional (2D) model. Important advantages of this model are its wide availability, ease of use and low costs, and a number of significant discoveries have been gained from its application (5). However, the artificial environment limits cell-cell interactions and thus prohibits a number of the physiological processes present in solid tumors (6). Furthermore, studies indicate that 2D cell cultures are a poor predictor for clinical benefits (7,8). These limitations are well-documented and the first attempts to mitigate these problems date back almost 40 years (9).

More sophisticated cell models were therefore developed. Multi-cellular spheroids were first described in 1970 with glioblastoma cells (9). These three-dimensional (3D) spheroids are considered to superiorly replicate the tumor environment, since spheroids allow cell-cell and cell-matrix contacts. As for the effectiveness of chemotherapeutic agents, the gradient of drug concentration in tumors plays an important role (10). Furthermore, the expression of surviving factors figures prominently in drug resistance (11). Studies have shown divergent response rates in colorectal carcinoma cells due to different protein expression profiles (11).

The extracellular matrix appears to be one significant factor for the resistance of a variety of cancer cells to radiation (12). Another significant factor for radioresistance is hypoxia in tumor cells. A lack of oxygen is often discussed as a mechanism for consecutive radioresistance and has been reported in human glioma cell lines (13).

In HNSCC, one important issue is the susceptibility of a patient to radiotherapy, since ~20% of patients only achieve

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Key words: head and neck squamous cell carcinoma, cell culture, multicellular spheroids, irradiation, cisplatin

a partial response or no response to irradiation (14). Another key problem is recurrence (15), since up to 50% of the patients are confronted with recurrent disease (16). The spheroid cell model may explain tumor biology more precisely and actualize therapy outcomes already in *in vitro* models. However, only limited data for 3D HNSCC cell models in comparison to 2D cell culture models is available (17-19).

Therefore, the present study sought to establish stable HNSCC spheroid protocols and accentuate its different features compared with 2D cell culture methods. Thus, the present study assessed the expression of important HNSCC proteins in the spheroids and compared the results with a 2D cell model. Ultimately, an established cytotoxicity protocol was established, and radiation and chemotherapy treatments were compared in each model.

Materials and methods

Cell Culture. The HNSCC SCC25, CAL27 and FaDu cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA) or the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The tumor cells were cultured as described previously (20). In brief, monolayer cells were grown in RPMI medium supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum (all Gibco BRL, Gaithersburg, MD, USA). Spheroids were grown in Dulbecco's modified Eagle's medium Ham's F-12 (Lonza, Verviers, Belgium) enriched with epidermal growth factor (EGF; Sigma-Aldrich, St. Louis, MO, USA), basic fibroblast growth factor (bFGF; Sigma-Aldrich) and B27 (Invitrogen Life Technologies, Carlsbad, CA, USA) supplement to conduce multicellular growth; these are commonly used supplements in free-floating spheroid models. The two cell models were cultured in a humidified 5% CO₂ atmosphere at 37°C.

Cytotoxicity assay. For the assessment of growth inhibition, cell counting kit 8 (CCK-8; Dojindo Molecular Technologies Inc., Gaithersburg, MD, USA) was used. For the 2D cell culture, the assay was performed according to the manufacturer's instructions. Hence, 3x10³ cells per well were added to 96-well plates (Sarstedt, Inc., Newton, NC, USA) and allowed to rest for 24 h. For the 3D cell culture, 2x10⁴ cells per well were seeded into Ultra Low Attachment plates (Costar, Corning Life Sciences, Tewksbury, MA, USA) and allowed to rest for 72 h. In each culture model, the cells were treated with increasing concentrations of cisplatin (0-20 μM) or radiation doses (0, 2, 4, 6 and 8 Gy) using a conventional radiation source with 150-kV X-rays (dose rate, 1 Gy/0.73 min). After 72 h of incubation, cell growth was measured. All experiments were carried out in triplicate and performed as at least three independent experiments.

Immunohistochemistry. In the 2D cell model, 7.5x10⁴ cells were spun onto microscopic slides using a Shandon Cytospin II centrifuge (130 x g for 3 min; Thermo Fisher Scientific, Waltham, MA, USA) fixated with acetone for 3 min at 4°C. For the 3D cell model, spheroids were grown as aforementioned. After 5 days, the spheroids were fixated in 8% formaldehyde solution for 30 min, and consecutively casted with 4% agarose gel and stored in phosphate-buffered saline at 7°C until paraffinization. Ultimately, tumor sections of a 2-3-μm thickness were created.

Immunohistochemical staining was performed as previously described (20,21). The three cell lines were stained for Ki-67, vascular endothelial growth factor receptor (VEGFR), EGF receptor (EGFR) and survivin. Ki-67 serves as a proliferation marker (22), VEGFR is an (lymph) angiogenesis receptor (23), EGFR is a member of the Her/erbB receptor family and an important receptor tyrosine kinase in HNSCC (24), and survivin is described as an apoptotic inhibitory protein (25). Primary antibodies for Ki-67 (monoclonal rabbit antibody; Abcam, Cambridge, MA, USA; dilution, 1:400), VEGFR (polyclonal rabbit antibody; Sigma-Aldrich; dilution, 1:100), EGFR (monoclonal rabbit antibody; Abcam; dilution, 1:100) and survivin (monoclonal rabbit antibody; Abcam; dilution, 1:500) were applied for 60 min. A polymer enhancer was used for 10 min prior to adding the high-resolution polymer (Thermo Fisher Scientific) for 15 min. Slides were developed using diaminobenzidine reagent (Thermo Fisher Scientific), counterstained with hematoxylin, dehydrated and mounted under a coverslip. Samples were analyzed using an Olympus BH-2 microscope (Olympus Corporation, Tokyo, Japan) and assigned to one of four categories of marker expression: 0, <5%; 1 (weak), 5-30%; 2 (moderate), 30-60%; and 3 (strong), 60-100%. An average expression score was calculated for every cell line. Experiments were repeated three times and histological analysis was performed by two independent investigators.

Statistical analysis. Data was analyzed by Student's t-test or one-way analysis of variance using SPSS software version 21 (IBM SPSS, Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference. All experiments were repeated at least three times. Error bars represent the standard error of the mean.

Results

Spheroid cell growth. Culturing of the HNSCC SCC25, CAL27 and FaDu cell lines according to the 3D protocol showed that all three cell lines exhibit certain degrees of spheroid growth. The FaDu cells showed the best cluster shape, cell-cell adherence and spheroid size (Fig. 1A). The SCC25 cells grew in denser and rounded clusters, whereas the CAL27 cells formed long and loose clusters (Fig. 1B and C).

Inhibitory concentrations of cisplatin. To establish a 3D cytotoxicity assay, different cell counts were seeded. After various incubation times (48 h for FaDu cells; 72 h for CAL27 and SCC25 cells), the cells were submitted to cisplatin treatment. The metabolic activity in the FaDu cell line was strongest as it led to an intense change of color in the CCK-8 assay. While the SCC25 cells showed less enzymatic conversion, the 3D culture of the CAL27 cells did not lead to feasible results in this assay. Hence, growth inhibition experiments were continued with FaDu and SCC25 cell lines.

In the 2D cell model, the cells were incubated with cisplatin concentrations ranging from 0-20 μM. In the two cell lines, cisplatin led to strong growth inhibition, with a half maximal inhibitory concentration (IC₅₀) of 0.95 μM in the FaDu cells and 3.26 μM in the SCC25 cells. By contrast, the spheroids were less susceptible to cisplatin, with an IC₅₀ of

Table I. Immunohistochemical analysis results showing average protein expression levels for Ki-67, VEGFR, EGFR and survivin in 2D and 3D cell models.

Parameter	SCC25	CAL27	FaDu	2D:3D	P-value
Ki-67					
2D	2.7	2.5	2.3	3.2	0.0134
3D	0.7	0.7	1.0		
VEGFR					
2D	2.5	2.8	2.0	1.2	0.7289
3D	3.0	1.5	1.5		
EGFR					
2D	2.8	1.0	2.3	0.7	0.4460
3D	3.0	3.0	2.8		
Survivin					
2D	2.8	2.8	3.0	1.1	0.0595
3D	2.6	2.6	3.0		

Samples were categorized to one of four expression levels: 0, <5%; 1 (weak), 5-30%; 2 (moderate), 30-60%; and 3 (strong), 60-100%, and then an average expression score was calculated. VEGFR, vascular endothelial growth factor receptor; EGFR, epidermal growth factor receptor; 2D, two-dimensional; 3D, three-dimensional.

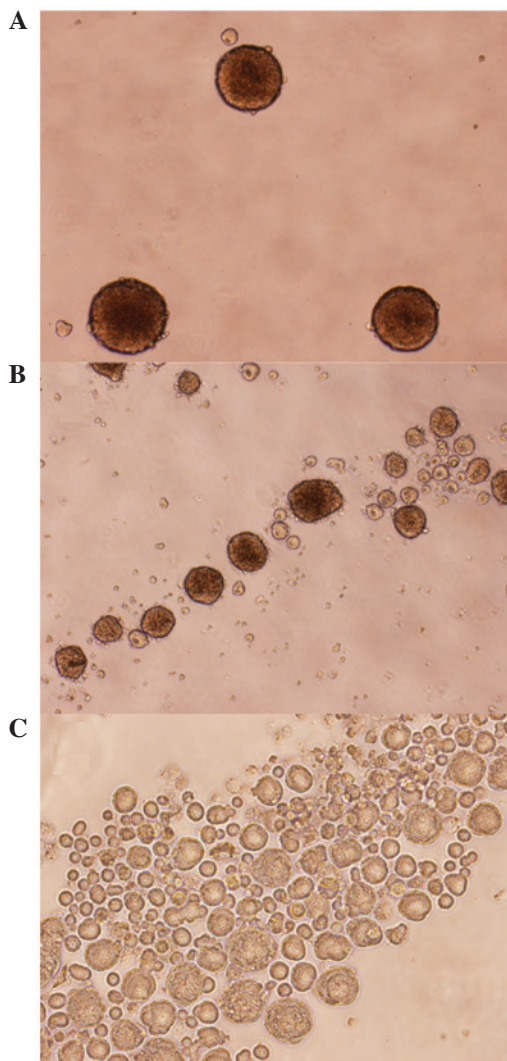


Figure 1. Microscopic images of 5-day-old spheroids of head and neck squamous cell carcinoma (A) FaDu, (B) CAL27 and (C) SCC25 cells (magnification, x40).

2.31 μM in the FaDu cells and 150.30 μM in the SCC25 cells. Dose-response curves for each cell line and culture model are shown in Fig. 2.

Response to radiation. The cells were exposed to increasing doses of radiation (0-8 Gy). Dose-response curves are depicted in Fig. 3. In the FaDu cell line, a comparable response to irradiation was observed in each cell model. However, in the SCC25 cells, the 2D cell model was significantly more susceptible to irradiation, since the spheroids showed no growth inhibition even after 8 Gy in the 3D model.

Immunohistochemical staining. The cell culture models were compared with regard to the expression of Ki-67, VEGFR, EGFR and survivin. Average expression scores were calculated and a 2D:3D ratio was established. Expression levels and ratios for all cell lines and proteins are summarized in Table I. With regard to Ki-67, moderate to strong staining was detected in the 2D model in all three cell lines, and the average staining score was three times higher than in the 3D cell model ($P=0.0134$). Staining was observed in a high percentage of cells. By contrast, only a few cells were stained with Ki-67 in the 3D model. The majority of the Ki-67-expressing cells were located in the outer third of a spheroid, solitary cells were observed in the intermediary zone and no Ki-67 staining was observed in the necrotic center (Fig. 4A and B).

The expression of VEGFR in the monolayer culture was likewise ubiquitous, although FaDu showed less staining. In the spheroids, VEGFR was expressed through all layers of the cell cluster. In the CAL27 cells, lesser staining was observed in the 3D cell model compared with the 2D model, whereas in the SCC25 and FaDu cells an inverse staining pattern was assessed (Fig. 4C and D). In the CAL27 cells, only weak staining of EGFR was observed in the monolayer model. However, in the 3D culture, the CAL27 cells strongly

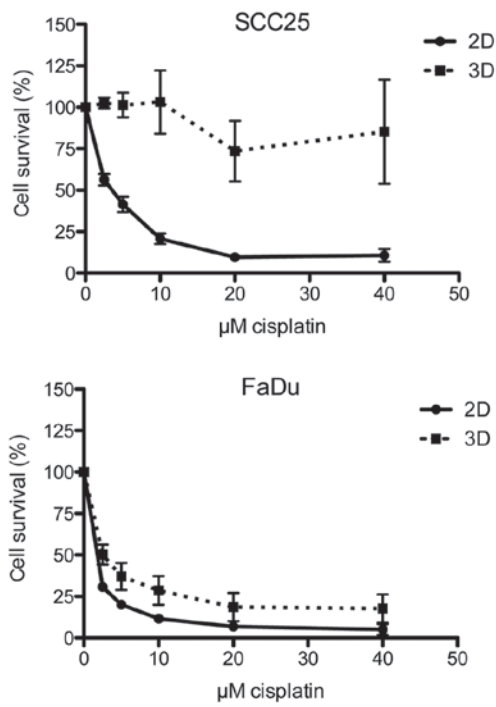


Figure 2. Dose-response curves of head and neck squamous cell carcinoma SCC25 and FaDu cell lines in the presence of increasing doses of cisplatin after 72 h of incubation. All experiments were performed in triplicate and repeated three times, and the data are presented as the mean and standard error of the mean. 2D, two-dimensional; 3D, three-dimensional.

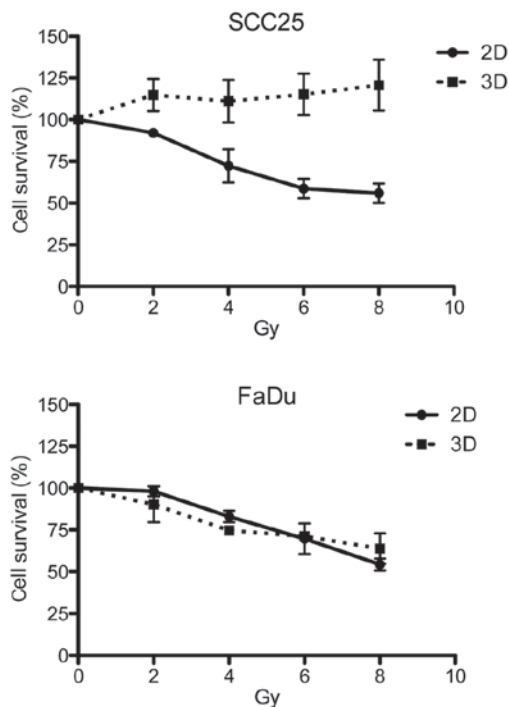


Figure 3. Surviving cell fraction after sole radiotherapy in the head and neck squamous cell carcinoma SCC25 and FaDu cell lines. The cells were treated with increasing doses of radiation ranging from 2-8 Gy. 2D, two-dimensional; 3D, three-dimensional.

expressed EGFR. In the FaDu and SCC25 cells, moderate to high expression was observed compared with high expression in the spheroids (Fig. 4E and F).

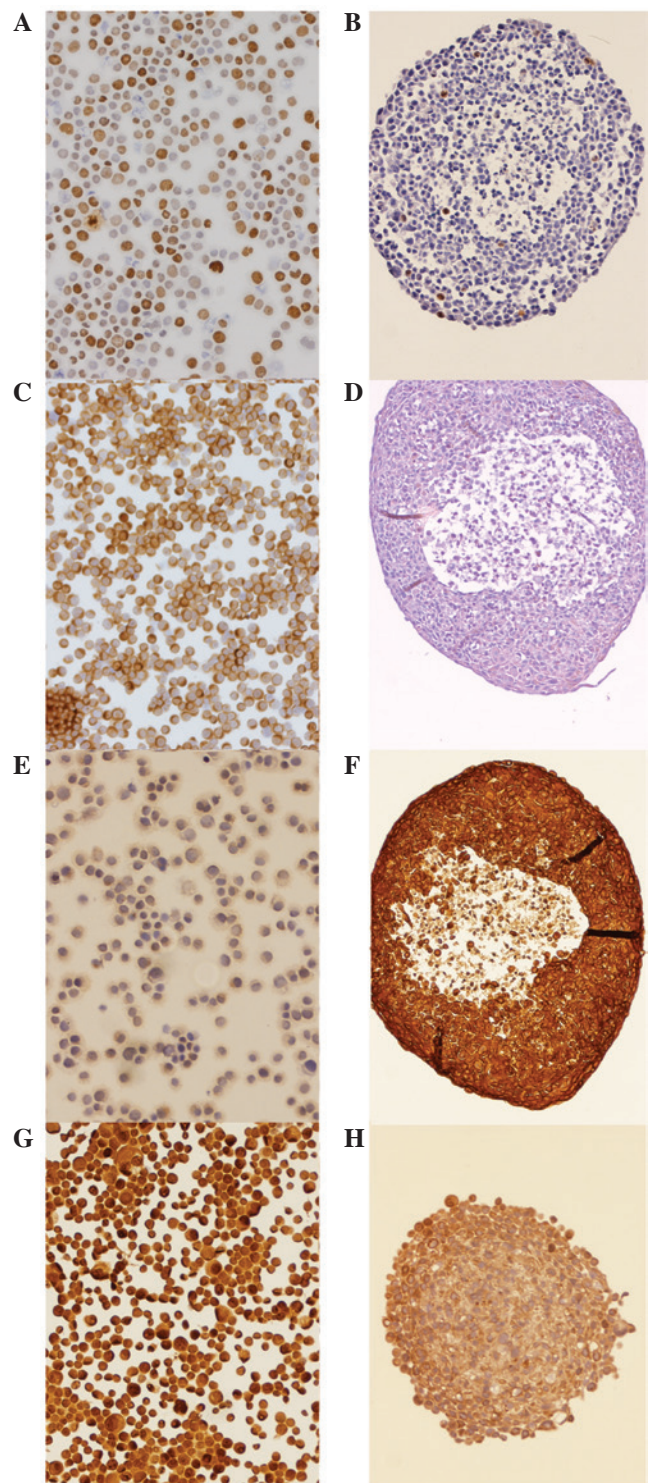


Figure 4. Immunohistochemical staining of head and neck squamous cell carcinoma spheroids 5 days after dissemination. (A) FaDu cells were spun onto slides, fixed and stained with Ki-67 in the monolayer model. (B) Staining of a spheroid (diameter, 300 μ m) of FaDu cells with Ki-67 antibody for the 3D model. Vascular endothelial growth factor staining of the CAL27 cells in (C) monolayer and (D) spheroid cell cultures. Epithelial growth factor staining in the CAL27 cells in the (E) 2D and (F) spheroid cell models. Survivin expression of the SCC25 cells in the (G) 2D and (H) spheroid cell models. All images were captured at x100 magnification.

With regard to survivin, no differential expression was observed in the 2D cell model compared with the 3D cell model (Fig. 4G and H).

Discussion

The capability of spheroid cell culture to reflect cancer biology more accurately *in vitro* has been described in various studies (4,11,26,27). Attempted explanations for the proximity of spheroid models to *in vivo* tumors in terms of proliferation and therapy response include cell heterogeneity and tumor microenvironment (28) or different levels of drug and oxygen diffusion (26).

To date, a certain number of studies on HNSCC spheroids have been published (29-46) but there is little knowledge on contraposing 2D to 3D cell cultures. These previous studies have focused on novel drug treatments or molecular expression patterns in 3D cell models. Therefore, although there is a broad knowledge regarding 3D cell cultures in HNSCC, a direct comparison between 2D and 3D cell cultures in HNSCC remains to be investigated. Hence, the present study aimed to establish a stable protocol for an HNSCC spheroid cell culture and to compare important attributes between the conventional monolayer and this spheroid cell culture. As expected from the previously mentioned studies, all three HNSCC cell lines, SCC25, CAL27 and FaDu, were able to grow in multicellular spheroids. Their growth patterns, however, differed between the cell lines and CAL27 did not exhibit reliable stability. Hence, this cell line was excluded from the cytotoxicity assays.

The capability of spheroid cell culture to better reflect cancer therapy resistance cultures has previously been described in hepatocellular (5) and colon (47) carcinoma. Since cisplatin is the main chemotherapeutic drug in the treatment of HNSCC, the present study exposed spheroids to cisplatin and compared the results to a monolayer cell culture. For the first time, a significant increase was observed in the IC₅₀ dose in one cell line, while comparable results were shown by each of the culture models in the other cell line. The site of origin may serve as an explanation, since SCC25 is derived from a tongue cancer and FaDu is from a hypopharyngeal tumor.

Since radiotherapy is a vital factor in HNSCC therapy and radioresistance is a determinative problem, the spheroids were irradiated. Notably, diverging growth inhibition was observed in the cell lines. In the FaDu cells, surviving fractions comparable to the monolayer cell culture were found, whereas in the SCC25 cells, the spheroids were significantly less affected by radiation than the monolayers.

Observations of resistance to irradiation have also been described in glioma cells (48). In the present study, an intermediate layer of hypoxia surrounding the center of spheroids was described; therefore, irradiation had less effect on spheroids. In addition, the protein expression of Ki-67 was examined within the spheroids. As expected, staining was concentrated at the periphery, since Ki-67 is considered as a proliferation marker (22). VEGFR was expressed throughout the whole spheroid. This finding is in contrast to that of a study by Shweiki *et al*, which described the weaker expression of VEGFR in the periphery of glial rat tumor spheroids (49).

However, comparing the location of primary expression in spheroids and the monolayer model is not possible, since 2D cells are prepared prior to the staining process. However, the staining intensity can be assessed and compared. In the present study, a variation in staining intensity was noted for Ki-67, VEGFR and EGFR, with partly stronger and partly weaker

expression in the spheroid cell model. Since VEGFR expression may result from hypoxia and cellular stress (50), lower expression was expected in the monolayer culture. However, only one cell line (CAL27) exhibited this pattern. Likewise, a similar expression pattern of EGFR was observed in the SCC25 and FaDu cells, whereas the CAL27 cells showed stronger expression in the spheroids. As for survivin, different staining intensities were expected, as described in a previous study (51). However, no distinction was possible between the spheroids and monolayer cells.

A spheroid cell culture was established from the HNSCC cell lines in the present study. The study described their response to cisplatin and radiotherapy for the first time. Despite being of the same cell line, different protein expression intensities and/or staining patterns were observed between spheroids and monolayer cell cultures. Hence, growth in spheroids alone alters the cellular pathways in the HNSCC cell lines. Thus, spheroid cell culture may prove to be the more rational alternative for cell culture due to the more physiological growth of the cells. Limiting factors, however, are higher costs, more elaborate maintenance and the impracticality of certain cell lines.

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