ORIGINAL ARTICLE

Putative cancer stem cells are present in surgical margins of oral squamous cell carcinoma

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Summary

Purpose: Recent evidence suggests that small subpopulations of stem-like cells are accountable for tumour initiation, progression and metastasis. Until now, studies were focused exclusively on the characterization of these cell populations within the tumour itself, while tumour margins were neglected, although it is known that the histological and molecular status of tumour margins may play a significant role in the course of the disease. Therefore, the aims of the study were to isolate cells from oral squamous cell carcinomas and their respective margins, to characterize these cells using specific markers, to assess their self-renewal potential and determine their chemoresistance.

Methods: Cell cultures were obtained from 12 tissue specimens (6 tumours and 6 margins). Total RNA was extracted and gene expression analysis was done by real-time PCR (RT-PCR). Flow cytometry, immunocytometry, sphere formation and MTT assays were also applied. **Results:** With minor differences, cells originating from both tumours and tumour margins showed the presence of stem cell markers CD133, Nanog, Sox2, CD44, and Oct4, had the capacity to form spheroids and showed chemoresistance.

Conclusions: Subpopulations of margin cells appeared to have stemness properties which might raise the question of re-evaluation of optimal surgical management.

Abbreviations: HNC-Head and neck cancer; OSCC- Oral squamous cell carcinoma; CSC- Cancer stem cell; DMEM-Dulbecco's Modified Eagle Medium; FBS- Fetal bovine serum; PBS- Phosphate buffered saline; TuP1- Tumour cells of the 1st passage; TuP5- Tumour cells of the 5th passage; MP1-Margin cells of the 1st passage; MP5- Margin cells of the 5th passage; OD- Optical density

Key words: cancer stem cells, embryonic and mesenchymal markers, oral squamous cell carcinoma, surgical margin

Introduction

Head and neck cancer (HNC) is the sixth most common cancer in developed countries with nearly 350,000 cancer deaths globally, per year [1]. The great majority of HNC are oral cancers (85%), mostly oral squamous cell carcinoma (OSCC). Despite significant advances in contemporary therapy, survival rates of patients with oral cancer have only moderately improved during the last 20 years [2,3]. Many attempts have been made to uncover new

biological markers that would improve our understanding of the natural history of OSCCs and thus provide more accurate diagnosis and prognosis and more favorable outcome for these patients. Despite the undisputable advances in OSCC understanding and treatment, it remains a considerable medical challenge. Recent evidence suggests that small populations of stem-like cancer cells are responsible for tumour initiation, progression and metas-

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tasis [2,4]. The so-called cancer stem cells (CSCs) have been identified, isolated, and characterized in various types of cancers, including HNC [3].

The main limitation of the previous studies of CSCs is the fact that they neglected tumour margins. It is well known that histological and molecular status of surgical margins is among the major factors affecting the rate of recurrences and metastases of OSCC [5-7]. Surprisingly, there are no studies dedicated to characterization of cells originating from tumour margins in terms of CSC markers. Therefore, the aims of this study were to culture cells from OSCC and its margin, characterize them in terms of stemness traits and evaluate the effect of serial passaging on cells phenotypic changes.

Methods

Cell culture

Tumour and margin tissues of six patients diagnosed with OSCC (4 males and 2 females, aged 59.5±9.33 years) were obtained from the Clinic of Maxillofacial Surgery of School of Dental Medicine, University of Belgrade, immediately after surgery. The study was approved by the Ethics Committee of the School (no. 36/31) and was in accordance with the Declaration of Helsinki. All patients were informed of the study and signed a written informed consent form. Three tumours were localized on the mouth floor and three on the tongue. Two patients were moderate alcohol consumers, whilst all of them were cigarettes smokers. One patient had stage I, one stage IVa with recurrences, and the remaining had stage II OSCC, based on TNM classification [8]. Surgical margins were taken 5 mm from the edges of the tumour, labeled by the pathologist as "cancer-free", as previously described [6]. The histopathological diagnosis was established according to the World Health Organization guidelines, using haematoxylin and eosin-stained tissue [8]. The SCC 25 cancer cell line (ATCC[®] CRL- 16 28[™] Rockville, MD), was used as control. Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% fetal bovine serum (FBS) and 100 U/ml penicillin-100 µg/ml streptomycin (Sigma- Aldrich, St. Louis, Missouri, USA) was used to transport the tissue samples. The tissue samples were minced with blades into 1 mm³ pieces and washed 3 times with phosphate buffered saline (PBS) to remove loosely bound cells. Explant-cell culture system [9] was carried out, with periodical removal of fibroblasts using differential trypsinization [10]. Cells were grown in DMEM supplemented with 10% FBS and 100 U/ml penicillin 100 µg/ml streptomycin, seeded onto T75 cell culture flasks (Figure 1). A 1:1 mixture of DMEM and Ham's F12 medium supplemented with 400 ng/ml hydrocortisone and 10% FBS was used for culturing SCC 25 cells. The cells were preserved at 37°C in humidified atmosphere containing 5% CO₂. The medium was changed every 2-3 days and the cells were passaged prior to reaching 80% confluence. Tumour (Tu) and margin (M) cells used in the present study were obtained after the 1st (P1) and the 5th (P5) passage. All experiments were done in triplicate.

Isolation of RNA and gene expression analysis

Total RNA was extracted from margin, tumour and SCC 25 cells (10⁶ cells per tube) using TRIzol (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA). Complementary DNA was prepared using Revert Aid First Strand cDNA Synthesis kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Subsequent RT-qPCR analysis was performed on Line Gene-K Fluorescence Real-time PCR Detection System (Bioer, China) using Maxima[™] SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific). The sequences of human specific primers used were as follows (5'to 3'): Oct4: forward GTGGAGAGCAACTCCGATG, reverse TGCTCCAGCTTCTCCTTCTC; Sox2: forward GACTTCA-CATGTCCCAGCACTA, reverse CTCTTTTGCACCCCTCC-CATT; CD44: forward AAAGCAGGACCTTCATCCCAGTGA; reverse ATTTCCTGAGACTTGCTGGCCTCT; CD133: forward ACTTGGCTCAGACTGGTAAA, reverse GTTCTGAG-CAAAATCCAGAG; Nanog: forward ATTCAGGACAGCC-CTGATTCTTC, reverse TTT TTGCGACACTCTTCTCTGC; GAPDH: forward TCATGACCACAGTCCATGCCATCA,



Figure 1. Representative images of tumor cells: **A:** 2-3 days after applying the tumor explant- cell culture system (the tumor fragment is marked with an asterisk *); **B:** after the fifth passage (100 x magnification).

reverse: CCCTGTTGCTGTAGCCAAATTCGT. The expression of GAPDH was used for normalization. The $\Delta\Delta$ Ct method was used for the relative quantification of gene expression [11].

Flow cytometry

Cells (7×10⁵ cells/ ml) were washed with PBS, labeled with monoclonal antibody CD44-APC conjugated (Exbio, Prague, Czech Republic), CD90-FITC conjugated (Life Technologies, CA, USA), CD73-PB conjugated (Sony Biotechnology, CA, USA), CD34-FITC conjugated (Sony Biotechnology, CA, USA) antibodies, incubated for 45 min, washed 3x with PBS and fixed with 2% formal-dehyde. The next day cells were subjected to analysis using flow cytometer (Partec, Munster, Germany). The

software used for the analysis was CyFlow Space (Sysmex Partec , Goerlitz, Germany).

Immunocytochemistry

Cells were seeded in 25 mm diameter round glass coverslips at density of 10⁴ cells/cm². After 24hrs, cells were rinsed 3 times in PBS, fixed with 4% paraformaldehyde for 20 min, and then rinsed 3 times with PBS and incubated at room temperature for 45 min in blocking and permeabilization buffer (10% bovine serum albumin and 0.1% Triton X-100 in PBS). Cells were incubated with CD44-APC conjugated monoclonal antibody (1:400) overnight at 4°C. Then, cells were washed 3 times in PBS and stained with 4-, 6-diamidino-2-phenylindole (1:4000, Molecular Probes, USA) for 10 min in the dark.



Figure 2. The level of expression of CSC markers in margin, tumor and control cell line cultures (* p<0.05; *** p< 0.001).

After washing in PBS, cell samples were mounted with Mowiol (Sigma-Aldrich) on microscope slides. Immunofluorescent images were obtained by confocal laserscanning microscope (LSM 510, Carl Zeiss GmbH, Jena, Germany) equipped with Ar 488 and HeNe 543 and 633 laser lines. Micrographs were analyzed using Fiji-Image J software (NIH, USA).

Spheroid formation assay

Cells were seeded at density of 10^4 /ml in 24-well culture plates previously coated with 1ml poly-HEMA (poly 2-hydroxyethyl methacrylate, Sigma-Aldrich) to prevent cell attachment as described previously [12]. After seeding, cells were incubated in DMEM supplemented with B-27, N₂ supplements, epidermal growth factor and antibiotics (Sigma-Aldrich). After 7 days of incubation, the number of spheroids per well was counted under microscope.

MTT assay

Ten thousand cells were seeded in a 96-well plate. After 24hrs, cells were treated with 100 μ l of 80, 50, 20 μ g/ml 5-fluorouracil (Medac, Wedel, Germany). After 24hrs, MTT (3-(4,5-dimethylthiazolyl-2)-2,5 diphe-



Figure 3. The percent of CD44, CD90, and CD73 positive cells **(a-c)** in margin, tumor and control cell line cultures (***p<0.001).

nyltetrazolium bromide) was added to each well, incubated for 4hrs, and the supernatant was discarded. Precipitates were dissolved in 100 µl dimethyl sulfoxide (DMSO) (Sigma-Aldrich) by shaking at 37°C. Optical density (OD) was measured at 540 nm using an ELISA reader (RT- 2100c, Rayto, China). The percentage of viable cells was calculated using the following formula: % of viable cells = OD (sample)/OD (control) x 100.

Statistics

Independent sample Student's *t*-tests were performed in the study. The values are presented as mean \pm SD. Statistical significance was set at p< 0.05. SPSS ver. 17 was used for the analyses (SPSS Inc, Chicago, IL, USA).

Results

Gene expression analysis

RT-qPCR analysis demonstrated the expression of markers related to cancer stem cells (CD133, Nanog, Sox2, CD44 and Oct4) in both tumour and margin cells. Sox2, Nanog and Oct4 were more upregulated in tumours than in margins but without statistically significant difference. The levels of expression of all stem/progenitor markers were higher in the 5th passage compared to the 1st (p <0.05) both in tumour and margin cells (Figure 2). TuP5 cells from the patient with stage IVa had the highest expression of CD44.

Flow cytometry

Flow cytometry analysis showed increased level of CD44, CD73, and CD90 in cultures of the 5th compared to cultures of the 1st passage in both tumour and margin cells (Figure 3). The difference between



Figure 4. Confocal microscopy of representative CD44 positive cells in tumor culture after the fifth passage (CD44 is red, nuclei are blue).

protein expression of tumour and margin cells was not statistically significant. Hematopoietic stem cell marker CD34 was negative in all the samples.

Immunocytochemistry

As expected, only a small portion of CD44⁺ cells was found. A representative image of tumour CD44⁺ cells is shown in Figure 4.

Spheroid formation assay

Cancer cell line, tumour, and margin cells (with the exception of MP1) had the ability to form spheroids (Figure 5). In the 5th passage tumours formed the highest number of spheroids (13.04), followed by SCC 25 (9.62) and margins (8.67), but without statistically significant difference.

MTT assay

The tumour cells of the 5th passage showed significantly higher chemoresistance compared to TuP1 (Figure 6). That trend was noted in the case



Figure 5. Representative images of **(a)** margin, **(b)** tumor after 1st passage, **(c)** tumor after 5th passage, and **(d)** SCC 25 spheres (100 x magnification).



Figure 6. The percentage of viable cells in margin, tumor and SCC 25 cultures after treatment with different concentrations of 5- fluorouracil (*p<0.05).

of margin cells as well, however without statistical difference. SCC 25 had the highest viability.

Discussion

In the present study, we successfully generated 12 cell cultures originating from 6 freshly resected OSCCs and their respective margins. We used early passages of the cell cultures to analyze subpopulations with stem cell features, assuming that early passages would reflect more accurately the situation in the patient.

The results of tumour cell cultures are in agreement with earlier findings on the existence of cells subpopulations with stemness traits within OSCC [3,13,14]. Yet, it must be emphasized that this is the first study to identify cells with stemness traits in OSCC margins. The potential existence of CSCs in seemingly normal surgical margins, might raise the question of re-evaluation of optimal surgical management. A 1-cm distant surgical margin [15] seems more adequate for surgical treatment of OSCC than the 5-mm used in the present study. Different levels of CD44, CD133, Oct4, Nanog and Sox2 mRNA were detected. CD44 antigen, a cell surface glycoprotein implicated in cell adhesion and migration, is considered to be one of the key CSC marker of head and neck SCC [3,16]. The expression levels of the aforementioned markers were not high, which can be explained by the fact that CSCs typically represent a small fraction of cells [3]. The expression of CD44 was the highest of all studied markers, which is in accordance with the first study on HNSCC stem-like cells [3]. Also, the patient with the most advanced stage of the disease (IVa) exhibited the highest level of this marker, in agreement with a recent study [17] which found a positive correlation between OSCC stage and CD44 expression level.

All of the studied markers showed higher expression in the 5th passage due to the fact that progressive cell culturing probably led to enrichment of the CSC population. Results obtained on SCC 25, which was a 10th passage cell line, confirm the trend of higher marker expression along with successive passages.

We further confirmed the presence of CSCs populations in tumour and margin tissue cultures using flow cytometry. Similarly to RT-qPCR analysis, flow cytometry showed increase of CD44⁺ cells of the 5th passage. Cells expressing CD90 and CD73, two mesenchymal stem cell markers, were also previously described in heterogeneous cancer cell populations [18].

Spheroid formation assay, based on self-renewal and growth capability of stem cells independent of anchorage, is considered as an *in vitro* surrogate for the *in vivo* tumour formation assay [19]. This assay was previously used to describe the presence of CSC in tumours [20], but never in surgical margins. In the present study, cells from both tumours and margins had the ability to form spheroids, thus confirming the existence of stem-like cells in margin cells subpopulation of the 5th passage. However, it must be emphasized that MP1 cells did not have the ability to form spheres which was one of the main differences of tumour and margin cells of the 1st passage. This ability appeared in MP5, which might suggest CSC enrichment during culture.

In the clinical settings, one of the most significant traits of CSC is chemoresistance [21]. It is also viewed as one of the main factors contributing to tumour recurrence after chemotherapy in many types of human cancers [22]. Tumour and margin cells displayed resistance to 5-fluorouracil that is frequently used as adjuvant therapy for OSCC patients [22]. Overall, cells of the 5th passage displayed higher resistance compared to cells of the 1st passage. This result can be related to the substantial overexpression of Oct4 and Nanog, as previously reported [23]. However, unlike margin,

tumour cells did not show dose-dependent sensitivity, suggesting higher chemoresistance.

In conclusion, to the best of our knowledge, this is the first time that cells showing CSC characteristics have been identified in surgical margins from OSCC patients, pointing to the need of reassessment of optimal surgical resection. Serial passaging appears to have enriched the CSC population in both tumour and margin cell cultures. Our work could represent a useful starting point for further studies on CSC identification and characterization in OSCC margins.

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Conflict of interests

The authors declare no conflict of interests.

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