Enhanced cisplatin resistance in oral-cancer stem-like cells is correlated with upregulation of excision-repair cross-complementation group 1

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Abstract  Background/purpose: Oral squamous cell carcinoma (OSCC) is a prevalent cancer worldwide. Recent data suggest that a subpopulation of cancer cells, termed cancer stem cells (CSCs), is capable of initiating, maintaining, and expanding the growth of tumors. Importantly, CSCs confer chemo- and radioresistance. Cisplatin is the most widely used chemotherapeutic agent, and chemoresistance to cisplatin is one of the major causes of tumor recurrence and metastasis with OSCC. However, the role of oral-cancer stem-like cells (OC-SLCs) in the chemoresistance of OSCC has not determined. The aim of this study was to investigate a key player of chemoresistance in OC-SLCs.

Materials and methods: OC-SLCs were isolated through sphere formation by cultivating the OC2 cell line in defined serum-free medium. Differential expression profiles of cell-surface stemness markers between enriched OC-SLCs and parental OSCC cells were elucidated by flow cytometry. Expression of excision repair cross-complementation group 1 (ERCC1) by OC-SLCs was examined by an RT-PCR and Western blotting.

Results: Initially, significant sphere formation (OC-SLCs) was observed in OC2 cells. Enriched OC-SLCs highly expressed stem-cell surface markers (CD117 and CD133) and the ABC transporter gene (ABCG2). Enhanced chemoresistance to cisplatin was also noted in OC-SLCs. Further, the chemoresistance of OC-SLCs to cisplatin was possibly correlated with ERCC1 upregulation in OC-SLCs.
Introduction

Oral squamous cell carcinoma (OSCC) is the sixth most prevalent malignancy worldwide and the third most common cancer in developing nations.\(^1,2\) A prognosis of OSCC remains dismal because >50% of patients die from this disease or complications within 5 years under current therapies.\(^3\) In the last several years, accumulating evidence has lent support to the idea that human cancer can be considered a stem-cell disorder.\(^4\) According to the hierarchical "stem-cell model of carcinogenesis," tumors are not to be viewed as simple monoclonal expansions of transformed cells, but rather as complex tissues where abnormal growth is driven by a rare cancer stem-cell (CSC) pool that acquires tumor-like features such as uncontrolled growth and also maintains its innate capacity to self-renew and differentiate into phenotypically heterogeneous progeny.\(^5\) CSCs are also generally believed to possess unique survival mechanisms.\(^6\) In view of their stem-like properties, these cells are believed to be responsible for maintaining and propagating the entire tumor.\(^7\) Therefore, to completely eradicate a tumor and prevent recurrence, it is imperative that CSCs be specifically targeted.\(^8\) In our previous study, we derived a subpopulation of oral-cancer stem-like cells (OC-SLCs) from OSCC with serum-free medium by sphere formation.\(^9\) The enriched OC-SLCs possess characteristics of both stem cells and malignant tumors.\(^9\) However, the function of OC-SLCs in chemoresistance in OSCC has not been determined.

Platinum-based chemotherapy has been used for treating a wide variety of solid tumors including lung, head and neck, ovarian, cervical, and testicular cancers for over 3 decades.\(^10\) Cisplatin predominantly interacts with DNA to form intra-strand crosslinked DNA adducts which trigger a series of intracellular events that ultimately result in cell death.\(^10\)–\(^13\) Although platinum therapy was a major advance in improving patient outcomes, the emergence of resistance limits the effectiveness of platinating agents in solid tumors, including OSCC.\(^10,11\) Molecular mechanisms of resistance are complex,\(^10,11\) but it is clear from recent studies that the DNA-repair capacity may have a major impact on resistance to cisplatin.\(^14,15\) DNA intra-strand crosslinks are processed and repaired by the nucleotide excision repair (NER) pathway in mammalian cells.\(^16,17\) To address these critical clinical issues, several potential predictive and prognostic markers were investigated. In those studies, the excision repair cross-complementation group (ERCC1), a critical protein within the NER pathway, was the focus. Evaluation of ERCC1 messenger (m)RNA levels in tumor samples taken from patients in small retrospective clinical trials of ovarian, colorectal,\(^19,20,21\) and non-small cell lung cancer showed an inverse correlation with either the response to platinum therapy or survival.\(^22\) Another study also showed a statistically significant survival benefit in patients with low levels of ERCC1 who had received platinum-based chemotherapy, compared to patients with high levels of ERCC1 who had received cisplatin chemotherapy.\(^23\)

Patients generally die of cancer after current therapies fail to eliminate residual disease. Due to the enhanced tumorigenicity of CSCs, it was proposed that failure to effectively treat cancer may be related to preferential resistance of these CSCs to chemotherapeutic agents. Evidence for chemoresistance by SLCs in epithelial cell lines and xenogeneic tumor-derived cells was presented.\(^24\)–\(^26\) Here, we demonstrated that the OC-SLCs generated from an OSCC cell line had enhanced chemoresistance to cisplatin, and the chemoresistance of OC-SLCs was possibly correlated with ERCC1 upregulation. Our results imply that mechanisms of resistance to platinum chemotherapeutic agents in OC-SLCs might be attributed to ERCC1 upregulation.

Materials and methods

Cell line and isolation of OC-SLCs from OSCC

OC2 is a cell line derived from an OSCC specimen of a buccal-mucosal squamous carcinoma from a Chinese man. OC2 cells are tumorigenic in nude mice.\(^27\) Originally, OC2 cells were grown in RPMI supplemented with 10% fetal bovine serum. The OC2 cell line was then cultured in tumor sphere medium consisting of serum-free Dulbecco’s modified Eagle’s medium (DMEM)/F-12, N2 supplement, 10 ng/ml human recombinant basic fibroblast growth factor (bFGF), and 10 ng/ml epidermal growth factor (EGF). Cells were plated at a density of 7.5 × 10^4 live cells/10 cm dish, and the medium was changed every other day until sphere formation was observed in about 4 weeks.\(^9,28,29\)

Fluorescence-activated cell sorting (FACS) analysis

For OC-SLC surface marker identification, a single-cell suspension from trypsinized spheres was stained with anti-CD133, CD117 (c-Kit), or ABCG2 and secondary FITC- or phycoerythrin-coupled antibodies (MACS) and analyzed with a FACS Calibur apparatus (Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

Reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA of parental oral-cancer cells or derived OC-SLCs was purified. Briefly, total RNA was obtained using
the Trizol reagent (Invitrogen, Carlsbad, CA, USA). A SuperScript one-step RT-PCR with the Platinum Taq system (Invitrogen) was carried out to analyze RNA molecules. Reverse-transcription was performed at 50°C for 30 minutes, and the PCR was performed with specific primers in 50 µl volumes according to the protocol provided by the manufacturer (Invitrogen). Endogenous ERCC1 RNA molecules were amplified using the forward primer, 5'-GATGCC AGAGACAGTGCCCCAAAG-3', and reverse primer, 5'-ACTA CACAGGCTGTCGGTGACCC-3'. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control in the RT-PCR with primers 5'-CCACATCGCTAGACACCAT-3' and 5'-CCCTCGACCTGCTTTACCAC-3'. The amplification reaction involved denaturation at 95°C for 15 seconds, annealing at 55°C for 30 seconds, and 72°C for 90 seconds using a thermal cycler (PTC-1000; MJ Research, St. Bruno, Quebec, Canada). The resultant RT-PCR products were analyzed on ethidium bromide-stained 1% agarose gels.

Western blot analysis

Cells were washed in phosphate-buffered saline (PBS) and incubated in ice-cold lysis buffer containing a protease inhibitor cocktail for 20 min. Equal amounts of protein samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a nitrocellulose membrane. The membrane was soaked in buffer (50 mM Tris-HCl and 0.15 M NaCl; pH 7.3) containing 5% low fat milk (Tris-milk) for 30 min with gentle shaking. A strip of a nitrocellulose membrane was incubated with a monoclonal antibody against ERCC1 (1:500 dilution in Tris-milk) for 30 min with gentle shaking. The strip was washed again, and the reactive bands were visualized using H2O2-chloronaphthol reagents. β-Actin was used as a loading control.

Cytotoxicity assays: the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cisplatin was obtained from Bristol Myers Squibb (Princeton, NJ, USA). Cell viability was evaluated using the an MTT conversion assay. Cells (10^4) were plated in 250 µl of medium/well in 96-well plates (Costar, Corning, Rochester, NY, USA). After overnight incubation, various concentrations of cisplatin were added. After 24 h, 40 µl of 5 mg/ml MTT was added per well. After 2 h of incubation, cells were lysed with 100 µl/well of dimethyl sulfoxide (DMSO), and incubated overnight. The absorbance was read at 550 nm in a microplate reader (Bio-Rad, city?, ST?, USA). Cell survival was expressed as the percentage of absorbance relative to that of untreated cells.

Statistical analysis

The Statistical Package of Social Sciences software (SPSS, Chicago, IL, USA) was used for the statistical analysis. An independent Student’s t-test was used to compare continuous variables between groups. The statistical significance level was set to 0.05 for all tests.

Results

Isolation and cultivation of OC-SLCs from OSCC cells

To investigate the existence of OC-SLCs in oral-cancers cells, OC2, an oral cancer cell line, was cultivated in defined serum-free medium with bFGF and EGF. After being cultured for 2 weeks, cancer cells gradually detached from the culture dishes, aggregated, and formed sphere-like bodies. Significant sphere-like bodies were observed after 3 weeks of serum-free medium culture (Fig. 1).

Characterization of progenitor/stem-cell properties in isolated OC-SLCs

To further characterize the progenitor/stem-cell properties of our enriched OC-SLCs, we used flow cytometry to detect expression profiles of progenitor/stem-cell surface markers. As shown in Fig. 2, we detected that the majority of isolated OC-SLCs were positively stained with CD133 and CD117 (c-Kit), both of which are specific cell-surface markers of normal/tumor stem cells. We also detected that most of the enriched OC-SLCs from OC2 cells were ABCG2-positive (Fig. 2). Around 50% of OC-SLCs derived from OC2 cells also stained positively for CD133, CD117, and ABCG2.

Enhanced cisplatin resistance of isolated OC-SLCs

We monitored the chemoresistance of parental OSCC cells and OC-SLCs by an MTT assay. We further tested common chemotherapeutic agents against OSCC including cisplatin. As presented in Fig. 3, compared to parental OSCC cells, OC-SLCs were significantly resistant to cisplatin, which is currently used for OSCC chemotherapy (*P < 0.05; Fig. 3).

Elevated expression of ERCC1 in OC-SLCs

To investigate key genes involved in mediating the chemoresistance properties of OC-SLCs, transcriptional and translational ERCC1 expressions were examined by an RT-PCR and Western blotting. Total RNA of parental cells and of sphere-like bodies enriched with 4 weeks of serum-free medium culture from the OC2 cell line was purified. The level of the ERCC1 transcript of enriched OC-SLCs significantly increased compared to that of parental OC2 cells (Fig. 4A). In accordance with the RT-PCR results, the Western blotting data also showed that the protein level of ERCC1 was upregulated in enriched OC-SLCs (Fig. 4B).

Discussion

While a revolution in therapeutic strategies yielding significant clinical responses measured in terms of tumor regression and disease-free survival occurred in recent decades, overall survival from OSCC has failed to substantially improve.30 The recent identification of CSC subpopulations with the unique ability to fuel tumor growth, may shed light on the disconnect between response rates and overall survival.31 These cells possess unique survival...
Figure 1  Sphere body formation in OC2. Oral cancer cell line OC2 was used and cultured in DMEM/F-12 serum-free medium with bFGF and EGF. After being in culture for 3 weeks, cancer cells gradually detached from culture dishes, aggregated, and formed sphere-like bodies (SB). Increase of the volume of sphere-like body from the OC-SLC was observed with longer cultivation (4 weeks). Bottom right, magnification.

Figure 2  Increased stem cell surface markers expression in OC-SLC. (A) Expression profiles of progenitor/stem cell-specific surface markers including CD133, CD117, and ABCG2 in parental cells or derived OC-SLC were analyzed by flow cytometry. Single-cell suspension from parental cells or derived OC-SLC was either stained with control IgG antibody or experimental antibodies including anti-CD133 (left panel), CD117 (middle panel), and anti-ABCG2 (right panel); (B) Around 50% of OC-SLC derived from OC2 cells stained positively with CD133, CD117 and ABCG2, respectively (*P < 0.05).
mechanisms and distinctive stem-cell properties, including the ability to self-renew and differentiate, and also exhibit a marked ability to proliferate following a prolonged period of quiescence. That is, therapies which fail to adequately target CSC populations, which represent a minority of most epithelial tumors, will fail to eliminate those cells capable of regenerating the tumor after therapy has ceased. Furthermore, familiarity with both the means of resistance to a particular chemotherapeutic agent and the phenotypic identity of cells that harbor resistance mechanisms should help facilitate the discovery of therapies better able to clear minimum residual disease and prolong overall survival.

By analogy to normal stem cells, CSCs are inherently resistant to chemotherapy through mechanisms that serve to protect stem cells from DNA and cellular damage. Firstly, most current agents destroy rapidly proliferating cells. Because CSCs, like all stem cells, are relatively quiescent, the current chemotherapeutic approaches may be ineffective. Secondly, stem cells tend to be more resistant to chemotherapeutic agents and radiation than more mature cell types from the same tissue are. This is believed to be due to the presence of multidrug resistance, antiapoptotic proteins, and enhanced DNA repair mechanisms. Since this appears to hold true for CSCs, we would predict that they would also display resistance to therapeutics.

Most of the knowledge of cancer gene expression was obtained using material from whole-tumor preparations. Establishing mechanisms of resistance to chemotherapeutic drugs can be difficult, especially with heterogeneous tumors. Because CSCs often make up only a tiny proportion of all cancer cells in a tumor, their genotype may easily be obscured by all of the other cancer cells when this methodology is utilized. It is necessary to isolate the CSC subpopulation from tumors and evaluate their gene expression to identify molecular pathways which are critical to the CSC population. Our previous study successfully enriched OC-SLCs, a subpopulation of CSCs, from OSCC cells through serum-free cultivation. With this in vitro culture condition which facilitates sphere formation with enhanced tumorigenicity, the study model was established and offers a new approach to characterizing underlying mechanisms of drug resistance.

The nucleotide excision repair (NER) pathway seems to be a key pathway involved in mediating resistance or sensitivity to platinum chemotherapeutic agents. The NER
pathway is a highly conserved DNA repair pathway which repairs DNA lesions which alter the helical structure of the DNA molecule and interfere with DNA replication and transcription. The ERCC1 protein plays a key role in the NER pathway. ERCC1 dimerizes with xeroderma pigmentosum complementation group F, and this complex is required for the excision of damaged DNA. A large body of preclinical evidence suggests that ERCC1 mRNA or protein expression levels may be correlated with cisplatin resistance in human cancer cell lines. Further studies also showed increased sensitivity to cisplatin after antisense RNA inhibition of ERCC1. Evaluation of ERCC1 mRNA levels in tumor samples taken from patients in small retrospective clinical trials of ovarian, colorectal, and non-small cell lung cancers also showed an inverse correlation with either the response to platinum therapy or survival. The work we present is novel in that, not only is it the first study showing the expression of ERCC1 in isolated CSCs, but it is also the first study showing cisplatin resistance in the OC-SLC subpopulation. These data imply that ERCC1 might play a pivotal role in mediating platinum chemoresistance in CSCs, where it acts to excise damaged DNA which would otherwise insult these crucial stem/progenitor cells.

In conclusion, we demonstrated that OC-SLCs display higher ERCC1 expression with the ability of enhanced cisplatin resistance. The resistance of in vitro chemotherapy is partially due to preferential activation of ERCC1 gene expression. A greater understanding of the characterization of the CSC population will shed new light on the underlying biological mechanism of this specific population and, thus, will ultimately lead to the development of more-specific therapeutic agents for treating this deadly cancer.

References