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Original Article

Combining microfluidic chip and low-attachment culture devices to isolate oral cancer stem cells

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Abstract *Background/purpose:* Cancer stem cells (CSCs) are widely recognized as key drivers of cancer initiation, progression, and therapeutic resistance. Microfluidic chip technology offers a promising approach for CSC isolation and study. This study investigated the efficacy of a microfluidic chip-based method for isolating single cells from oral cancer cell lines characterized by high stem-like phenotypes. Specifically, the study focused on examining the sphere-forming capability and the expression of CSC markers, including aldehyde dehydrogenase 1A1 (ALDH1A1), CD44, and CD133, in isolated cell clones from OECM-1 and SAS cell lines.
Materials and methods: Oral cancer cell lines were subjected to isolation using a microfluidic chip. The captured single cells were cultured to assess their sphere-forming capacity in ultra-low binding culture. Furthermore, the protein expression levels of ALDH1A1, CD44, and CD133 in the isolated cell clones were analyzed using western blotting.
Results: The microfluidic chip-assisted isolation method significantly enhanced the sphere-forming capability of both OECM-1 and SAS cell clones compared to their parent cell lines. Moreover, the expression levels of CSC markers ALDH1A1, CD44, and CD133 were upregulated in the microfluidic chip-assisted isolated cell clones, indicating a higher stem-like phenotype.
Conclusion: This study demonstrates the effectiveness of the microfluidic chip-based approach in isolating oral cancer cell clones with elevated stem-like characteristics. This method offers

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a valuable tool for further investigation of CSCs and their role in cancer progression, as well as future therapy development for oral cancers.
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Introduction

Cancer stem cells (CSCs) are a subpopulation of cells within tumors that possess stem cell-like properties, including self-renewal and differentiation capabilities. CSCs are thought to play a critical role in tumor initiation, growth, and therapy resistance.[1] Studying CSCs is important for developing targeted therapies that specifically target these cells, which may be more effective than traditional therapies that target all cells within the tumor. CSCs have been identified in various types of cancer, including oral cancer. Targeting high stem-like phenotypes in oral cancer cell lines is highly relevant in understanding tumor progression and therapy resistance. CSC markers serve as essential agents for identifying cancer cells in precision anti-cancer therapies and improving chemotherapy efficiency. They have the potential to be exploited as indicators for preneoplastic lesion malignancy, oral cancer progression, and treatment prognosis. Various markers for head and neck cancer (HNC) and oral cancer stem cells have been identified and studied. Some of the commonly studied CSC markers in oral cancers include CD44, CD24, CD117, CD133, CD147, Oct4, aldehyde dehydrogenase (ALDH), and other pluripotency-associated genes.[2—4]

Isolation of CSCs can be achieved through various methods, including surface marker-based approaches and functional assays.[5,6] Microfluidic technology is an efficient tool for the isolation and characterization of stem cells, including CSCs.[7] Microfluidic devices can create highly controlled microenvironments for single-cell studies, enabling observations of cells under specific conditions and durations. This precision is critical for understanding cell behaviors and interactions in detail, making them a valuable tool for single-cell analysis. Along with their capacity to efficiently assess numerous samples, thereby facilitating comprehensive investigations of complex systems and cell populations in biology.[8] The other benefits of microfluidic systems also include precise control over small volumes of fluids, enhanced cellular analysis, and low reagent consumption.[9] Furthermore, microfluidic platforms exhibit enhanced operational control and reduced risk of contamination, leading to more efficient high-throughput experiments, and cost savings due to minimal quantities needed for microenvironment formation.[10] Remarkably, microfluidic platforms enable real-time on-chip analysis, extending even to single-cell levels.[11]

This innovative approach represents a significant advancement, enabling researchers to gain a deeper understanding of the fundamental characteristics of cancer cells. However, there is still a lack of study on harnessing microfluidic platforms in the isolation, culture, and analysis of oral CSCs. Conducting microfluidic applications to oral disease studies can hold the promise of improving diagnosis, treatment, and patient care in oral health practice. Thus, in this study, we present a pipette-based microfluidic cell isolation technique that is capable of conducting the isolation of single cells from oral cancer cell lines with elevated stem-like attributes. Specifically, the study focused on examining the sphere-forming capability and the expression of CSC markers, including aldehyde dehydrogenase 1A1 (ALDH1A1), CD44, and CD133 (prominin-1), in isolated cell clones from OECM-1 and SAS cell lines.

Materials and methods

Cell isolation and culture using microfluidic chip

Human oral squamous cell carcinoma (OSCC) cell lines HSC3, OECM-1, and SAS were used in this study. HSC3 is a cell line established from tumors of metastatic lymph nodes that originated in tongue squamous cell carcinomas of a 63-year-old male patient. OECM-1 cell line was isolated from gingival squamous cell carcinoma of male Taiwanese. SAS is a tongue squamous cell carcinoma cell line of female Asian patients. Single-cell isolation and culture from parental HSC3, OECM-1, and SAS cell lines were performed using a microfluidic chip (CellGem®, OriGem Biotechnology Inc., Taichung, Taiwan). The procedure followed the manufacturer's instructions, including three major steps (Fig. 1).

Step 1: Cell isolation and single-cell capture

Pre-rinse. Slowly add 1 mL of a priming solution composed of 30～35% ethanol into either inlet of the microfluidic (MF) chip. Maintain the solution still for 10—30 s, ensuring even distribution across the chip. Inject the priming solution rapidly three times to displace any trapped air bubbles and ensure complete filling of the chip's microstructures. Replace the priming solution by injecting 1 mL of phosphate-buffered saline (PBS) into the chip three times, ensuring complete replacement while avoiding bubble formation.

Cell loading and washing: Introduce 600 μL of cell suspension (10^6 cells/ml) into the microfluidic chip. Allow a 3-min incubation period to facilitate the settling of cells into designated capture wells. Inject 1 mL of PBS into one inlet of the chip, followed by another injection into the opposite inlet. Wait for 2 min to complete the washing process. Substitute the PBS with 1 mL of culture medium to create a conducive environment for subsequent steps.

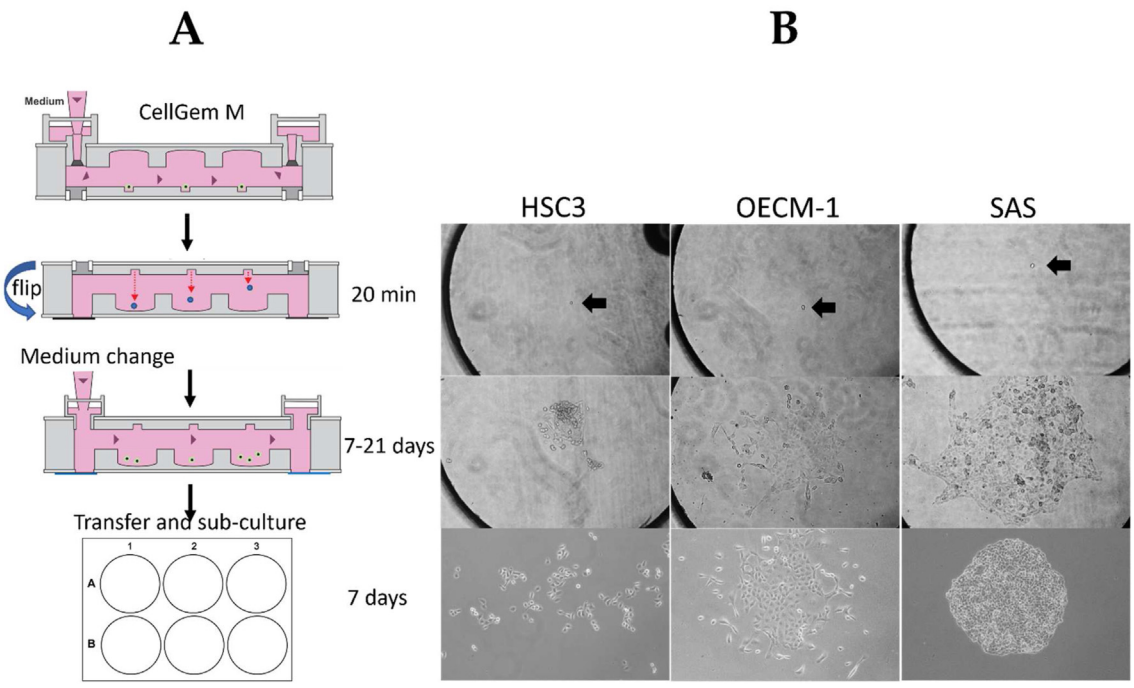

Figure 1 **A.** Schematic illustration of the procedure for OSCC cell isolation and culture using the microfluidic chip. **B.** Representative images of HSC3, OECM-1, and SAS cell clones from single cells (top) to the colony of cells in the culture well (middle) and in the 6-well culture plate (bottom).

Seal and flip. Aspirate any remaining liquid in the chip's reservoirs and remove the reservoirs from the chip. Clear any liquid from the chip's surface and refill the inlets with culture medium. Seal the inlets using sealing tape and flip the chip 180°. Maintain this position for 20 min, allowing cells to naturally settle into culture wells. To preserve culture humidity, add 2 mL of 1X PBS into the reservoirs located at the carrier's edge. Cover with lid and put the carrier with the chip in an incubator.

Step 2: Cell culture
After 1—2 days, when cells were attached to the bottom, cell culture reservoirs were installed, fresh medium was gradually injected into one of the reservoirs and hydrostatic pressure differences facilitated gradual medium replacement. Change the medium every 2—3 days.

Step 3: Cell harvest
After 7—21 days of culturing, the cultured cells were ready to harvest. The chips were gently disassembled to expose the culture wells, and 1.5 μL of trypsin was injected into each culture well to dissociate cells from the well bottom. The cells were then aspirated from the culture wells using a 2 μL pipet and transferred to an ultra-low binding culture plate.

To measure the efficiency of single-cell capture and the ability of isolated single cells to form cell clones using this CellGem microfluidic chip, we calculated as following:

Capture rate (%) = (number of capture wells trapping single cells/total number of capture wells) x 100.

Formation (growth) rate (%) = (number of culture wells having cell colonies formed/number of single cells captured) x 100.

Because a single capture well was only able to capture one single cell, the number of single cells captured was also the number of capture wells trapping single cells.

Sphere formation assay

The harvested cells were cultured in DMEM/F-12 medium supplemented with 1% penicillin-streptomycin-amphotericin B, 1% N2 supplement (Gibco BRL, Gaithersburg, MD, USA), 10 ng/mL epidermal growth factor (R&D Systems, Minneapolis, MN, USA), 10 ng/mL basic fibroblast growth factor (R&D Systems), 10 μg/mL insulin (Sigma-Aldrich, St. Louis, MO, USA) and 1 μM dexamethasone (Sigma-Aldrich). To initiate the sphere formation assay, OECM-1 and SAS cells were seeded at a density of 10,000 cells/ml into ultra-low binding 6-well plates (Alpha PlusS, Omics Bio, New Taipei, Taiwan). After 10 days of incubation, the sphere formation capacity of the cells was assessed by measuring the number of spheres (>75 μm) formed and sphere size (diameter in μm) under a microscope.[12]

Western blot analysis

Tumor spheres from non-adherent cultures were collected for protein extraction and western blot. The protocol was described in previous research.[13] Briefly, cell lysates were denatured using 5x SDS sample loading buffer at 95 °C for 5 min and loaded onto 10% SDS-PAGE gels with approximately 10 μg of total protein per lane. The separated proteins were transferred onto Polyvinylidene difluoride (PVDF) membranes, and subsequent blocking was performed with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween 20 for 1 h at room temperature.

Primary antibodies targeting ALDH1A1 (1:2000, #15910-1-AP, Proteintech Group, Rosemont, IL, USA), CD44 (1:2000, #15675-1-AP, Proteintech Group), CD133 (1:2000, #18470-1-AP, Proteintech Group), and GAPDH (1:10,000, NB300-221, Novusbiologicals, Centennial, CO, USA) were incubated with the membranes overnight at 4 °C. Subsequently, the membranes were exposed to secondary antibodies: anti-rabbit IgG, horseradish peroxidase (HRP)-conjugated antibody (1:30,000, Jackson ImmunoResearch, West Grove, PA, USA), and anti-mouse IgG, HRP-conjugated antibody (1:30,000, Jackson ImmunoResearch) for 1 h at room temperature. Immunoreactivity was detected using a chemiluminescent HRP substrate (Millipore, Burlington, MA, USA). The blot signals were digitally captured using the Fusion Solo image system (Vilber Lourmat, Marne-la-Vallée, France). Quantification of results was carried out using ImageJ software (National Institutes of Health, Bethesda, MD, USA), with signal normalization performed relative to GAPDH, serving as the loading control.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism (v9, GraphPad Software Inc., La Jolla, CA, USA). Unpaired parametric comparisons were executed using Welch's t-test, with a *P*-value of 0.05 or less indicating statistical significance.

Results

Capture rates and clone formation rates

Single-cell capture rates and cell clone formation (growth) rates of OSCC cell lines using the microfluidic chip were observed as follows (Table 1): In the HSC3 cell line, the capture rate was 60%, while the cell clone formation rate was remarkable at 72.2%. Similarly, in the OECM-1 cell line, the microfluidic chip achieved a capture rate of 65%, while the formation rate in OECM-1 cell clones was substantial at 61.5%, indicating the chip's proficiency in single-cell isolation and clone expansion. In the SAS cell line, the capture rate and clone formation rate were 63.3% and 42.1%, respectively. In summary, the average capture rate and formation rate were 62.8 ± 2.1% and 58.6 ± 12.5%, respectively. These results affirm the microfluidic chip method's suitability for single-cell isolation and subsequent cultivation in OSCC cell lines.

Enhancement of the sphere-forming capability of OSCC cell clones isolated by microfluidic chip

Because HSC3 has poor proliferative and sphere-forming capabilities in serum-free culture conditions, as reported by Ohnishi et al., only OECM-1 and SAS cell lines continued to be included in further investigations.[14]

Our data showed the utilization of the microfluidic chip (MFs groups) yielded significant outcomes regarding sphere formation in both the OECM-1 and SAS cell lines. Specifically, in the OECM-1 MF cell clones, the number and size of spheres exhibited a significant increase when compared to the parent OECM-1 cell population (Control group) (Fig. 2A). A similar trend was observed in the SAS cell line (Fig. 2B), where the number and size of spheres were significantly elevated in the MF chip-assisted isolation clones compared to the SAS parental cell line (Control group). These findings indicate the MF chip's efficacy in isolating cells with high stem-like characteristics, as demonstrated by the enhanced capacity of sphere formation in both OECM-1 and SAS cells.

Upregulation of CSC markers ALDH1A1, CD44, and CD133 protein expressions in OSCC cell clones isolated by microfluidic chip

ALDH1A1, CD133 (prominin-1), and CD44 are biomarkers that play a crucial role in identifying and understanding the characteristics of CSCs in various cancers, including oral cancers, which are accountable for tumorigenesis, self-renewal, and therapy resistance. A systematic review from Singh et al. reported that the three markers CD44, CD133, and ALDH were the most commonly expressed CSC markers in head and neck squamous cell carcinoma.[15]

In the OECM-1 cell line, while ALDH1A1 protein expression analysis showed only MF2 clone had a significant increase compared to the parent OECM-1 cells, a pronounced elevation in both CD44 and CD133 protein expressions were observed in all MF chip-assisted isolation clones (Fig. 3A).

Additionally, in the SAS cell line, all microfluidic chip-assisted isolations (MFs groups) yielded a remarkable increase in ALDH1A1 and CD133 protein expression compared to the parent SAS cell population (Control group). Meanwhile, CD44 protein expression also exhibited a significant increase in the MF2 and MF3 cell clones compared to their parent SAS cells (Fig. 3B).

The results show that the microfluidic chip-assisted isolation method was able to isolate cells from the OECM-1 and SAS cell lines that had higher expression of the stem cell markers ALDH1A1, CD133, and CD44 than the parent cell populations.

Discussion

In this current study, we used the microfluidic chip method for isolating CSCs from the OSCC cell lines. When compared to the parent cell populations, the microfluidic chip-assisted isolation cells displayed a significant increase in

Table 1 Capture rates and growth rates of OECM-1 and SAS cell clones isolated using microfluidic chip.

	HSC3	OECM-1	SAS
Capture rate	(36/60) = 60%	(39/60) = 65%	(38/60) = 63.33%
Growth rate	(26/36) = 72.22%	(24/39) = 61.53%	(16/38) = 42.10%

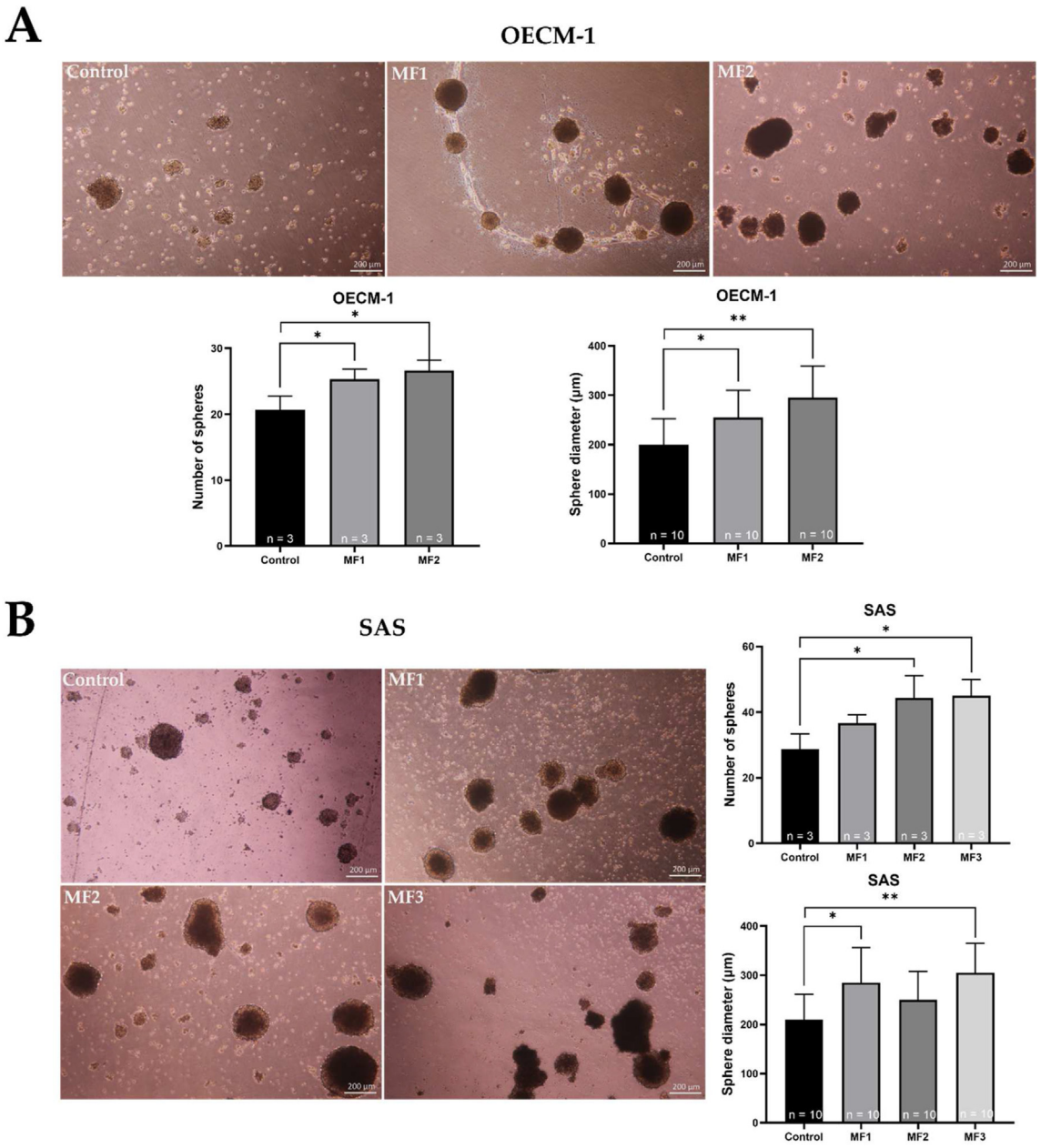

Figure 2 Enhancement of the sphere-forming capability of OSCC cell clones isolated by microfluidic chip. **A.** Representative images of tumorspheres from OECM-1 cell clones isolated by microfluidic chips (MFs) and their parental cells (Control). Quantification of the number and size of spheres formed by each cell line is shown in the charts below. **B.** Representative images of tumorspheres from SAS cell clones isolated by microfluidic chips (MF groups) and their parental cells (Control group). Quantification of the number and size of spheres formed by each cell line is shown in the charts on the right. Scale bars 200 μm. Data are expressed as mean ± SD. n, sample size; *$P < 0.05$, **$P < 0.01$, Welch's t-test.

the quantity and size of sphere formation, as well as the expression of CSC markers ALDH1A1, CD44, and CD133. ALDH is an enzyme that is involved in the metabolism of aldehydes. The role of ALDH1A1 in tumorigenesis could be due to its maintenance of CSC properties, modification of metabolism, and promotion of DNA repair.[16] Overexpression of ALDH1A1 was reported to be correlated with more advanced TNM tumor stages and nodal stages, reinforcing its role as an indicator of invasiveness.[17] On the other hand, CD133, a transmembrane glycoprotein, has been used as a stem cell biomarker for the isolation of stem-like cells and also serves as a significant cancer stem cell marker in OSCC. A study on OSCC cell lines reported that the CD133-positive side population has enhanced tumor sphere-forming ability, and silencing CD133 amplifies chemotherapy efficiency, suggesting the significant role of CD133 in tumor initiation and chemotherapy resistance in OSCC.[18] CD44 is a multifunctional cell surface glycoprotein involved in several cellular processes, including cell adhesion, migration, and proliferation. CD44 is overexpressed in many types of

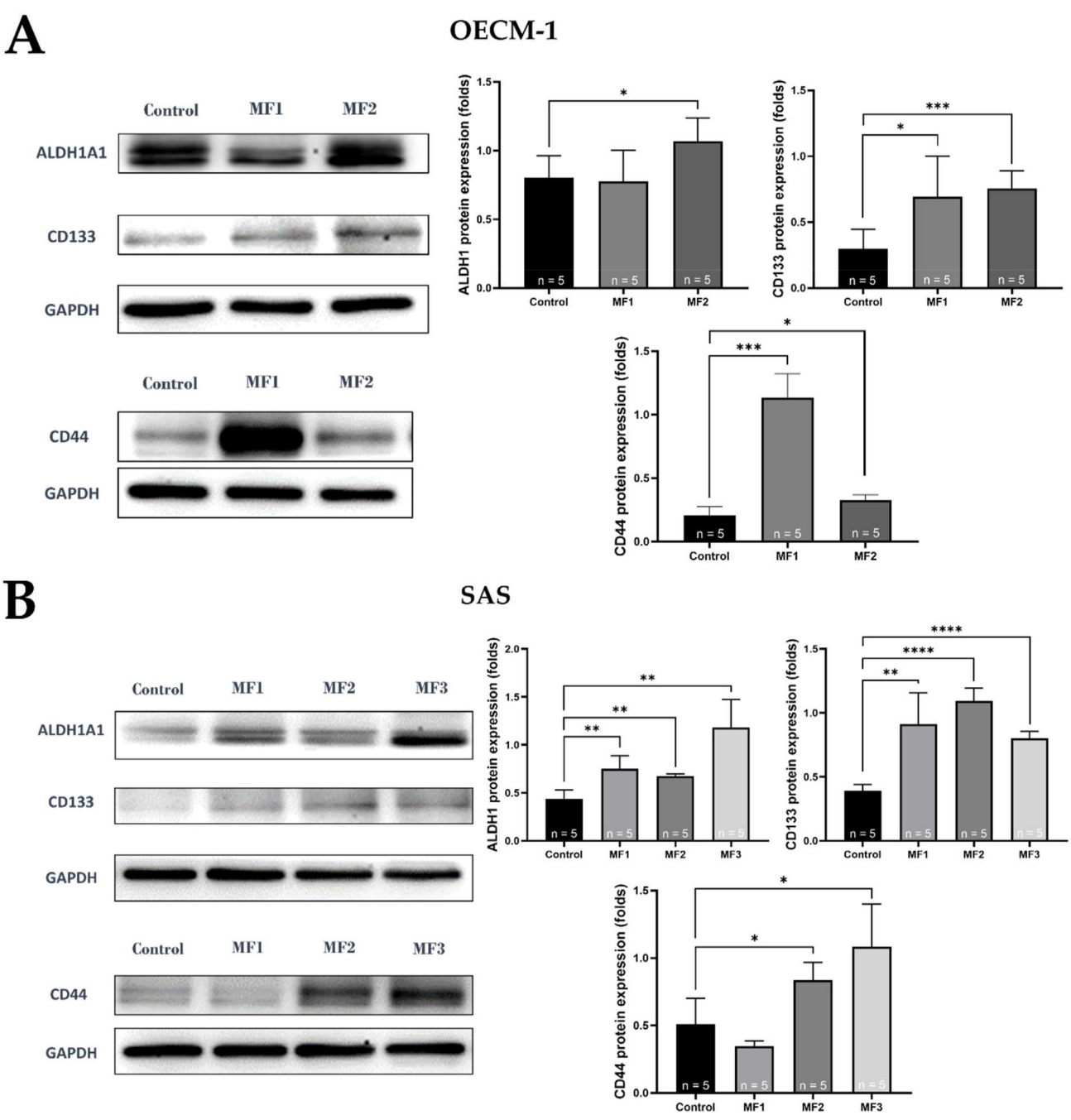

Figure 3 Upregulation of CSC markers protein expressions in OSCC cell clones isolated by microfluidic chip. ALDH1A1, CD44, and CD133 protein expressions in different clones of OECM-1 (**A**) and SAS (**B**) cell lines were investigated by western blot. MFs groups: Cell clones isolated by microfluidic chip; Control group: the parental cell line. The charts on the right show the quantification of protein expression levels, normalized to the loading control (GAPDH), in each group. Data are expressed as mean ± SD. n, sample size; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. Welch's t-test.

cancer, and it is thought to play a role in cancer metastasis.[19,20] Studies revealed that CD44 promoted chemoresistance. It was also related to the genetic process called epithelial-to-mesenchymal transition, which is associated with metastasis, thus contributing to tumor aggressiveness in oral cancers.[21–23]

The substantial elevation in ALDH1A1, CD133, and CD44 expression across microfluidic chip-assisted isolation OECM-1 and SAS cell clones indicates the high efficiency of this method in isolating CSCs from oral cancer cell lines. This uniform increase suggests the microenvironment provided by the microfluidic device's capability of contributing to the capture or enrichment of cells with stem-like properties.

Microfluidic chips can be used to isolate cells in a high-throughput manner, which means that a large number of cells can be isolated in a short amount of time.[24] This is

important for studying cancer stem cells because they are a small subpopulation of cells within a tumor. By using microfluidic chips, researchers can isolate enough cells to study their biology in detail. Furthermore, microfluidic applications can be used for rare cell capture, which is not only helpful in isolating CSC but also critical for analyzing disease progression.[25] In addition, microfluidic platforms require smaller sample volumes, making them ideal for working with precious biological specimens, like patient-derived samples.[26] This not only conserves samples but also reduces the need for extensive cell culture, saving both time and resources. Additionally, one of the key advantages of microfluidic chips is their ability to isolate cells with minimal damage.[27] The reduced cell damage is important in the study of fragile and sensitive cell populations, such as stem cells or primary cells. Cells isolated with minimal damage are more likely to yield high-quality data as their genetic and proteomic profiles remain closer to their in vivo states, which can help preserve the analytic accuracy of isolated cells. This is critical in cancer research as well as in clinical application. Moreover, the microfluidic device's capability of providing a controlled microenvironment for single-cell culture is essential for establishing and maintaining clonal cultures, which can be used to study clonal expansion and early neoplastic progression.[28] Clonal expansion in cancer is the process by which tumors arise from the expansion of a single or a small number of genetically or epigenetically altered cells.[29,30] Establishing single-cell or stem cell clones from primary OSCC tumors would be a valuable tool for studying the clonal expansion in OSCC tumorigenesis. By studying single-cell clones from primary OSCC tumors, we can investigate the clonal evolution of OSCC and identify the clones that are responsible for tumor progression and metastasis. Furthermore, by identifying the key genetic and molecular changes that drive the growth and survival of OSCC clones, we can develop new drugs that specifically target these clones.

In addition, microfluidic systems are designed to operate with reduced reagent volumes.[31] This is significant because it not only conserves expensive reagents but also contributes to a more sustainable and environmentally friendly research approach. Some digital microfluidic systems have been developed to provide precise control over the microenvironments in which cells are investigated. These platforms also allow for real-time monitoring of the target cell behavior.[11] This capability is valuable for studying CSCs and their dynamic processes like migration, differentiation, and responses to therapy. Finally, while setting up microfluidic systems may require an initial investment, the long-term benefits, including reduced reagent costs and increased experimental throughput, can lead to cost savings over time.

The microfluidic device that we used in our study is a commercial device that is available on the market. It is a relatively affordable and easy-to-use device that does not require any specialized training to operate. As aforementioned, this microfluidic device can isolate single cells in a high-throughput manner and provides a controlled environment for single-cell isolation, which reduces the risk of contamination and other errors, thus making it more efficient and reliable. This is in contrast to other methods, such as ring cloning and limiting dilution cloning, which are more time-consuming and labor-intensive.[32] Moreover, the microfluidic chip is versatile; it can be used to isolate single cells from a variety of cell types, including stem cells, cancer cells, and immune cells. Meanwhile, other methods are more limited in their applications, such as ring cloning, which is only applicable to adherent cells.[33]

Overall, this current study deployed a microfluidic chip approach for isolating CSCs in a simple-operative and cost-effective way, which achieved promising results in cancer stem-like phenotypes. However, there were limitations in this present study, which was only conducted on a limited number of oral cancer cell lines, necessitating broader assessments across various HNC cell lines. Moreover, although the study evaluated key CSC markers, the inherent heterogeneity of CSCs may demand more extensive marker panels. Nevertheless, our results provide evidence to support this microfluidic method as a useful tool for isolating and studying stem cells in oral cancer. Further studies are needed to confirm these results and to develop new methods for oral cancer therapy research based on this approach.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

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References

1. Bao S, Wu Q, McLendon RE, et al. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature* 2006;444:756–60.
2. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: globocan estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA A Cancer J Clin* 2018;68:394–424.
3. Ferlay J, Colombet M, Soerjomataram I, et al. Estimating the global cancer incidence and mortality in 2018: globocan sources and methods. *Int J Cancer* 2019;144:1941–53.
4. Chan CY, Lien CH, Lee MF, Huang CY. Quercetin suppresses cellular migration and invasion in human head and neck squamous cell carcinoma (HNSCC). *Biomedicine (Taipei)* 2016;6:15.
5. Hsu WL, Yu KJ, Chiang CJ, Chen TC, Wang CP. Head and neck cancer incidence trends in Taiwan, 1980 ~ 2014. *Int J Head Neck Sci* 2017;1:180–90.
6. Liu FH, Huang JY, Lin C, Kuo TJ. Suicide risk after head and neck cancer diagnosis in Taiwan: a retrospective cohort study. *J Affect Disord* 2023;320:610–5.
7. Aghlmandi A, Nikshad A, Safaralizadeh R, Warkiani ME, Aghebati-Maleki L, Yousefi M. Microfluidics as efficient technology for the isolation and characterization of stem cells. *Excli j* 2021;20:426–43.

8. Wu YH, Lai CH, Chien L, et al. Economic burden of cervical and head and neck cancer in Taiwan from a societal perspective. *Int J Environ Res Publ Health* 2023;20:3717.
9. Halldorsson S, Lucumi E, Gómez-Sjöberg R, Fleming RMT. Advantages and challenges of microfluidic cell culture in polydimethylsiloxane devices. *Biosens Bioelectron* 2015;63:218—31.
10. Wu H, Zhu J, Huang Y, Wu D, Sun J. Microfluidic-based single-cell study: current status and future perspective. *Molecules* 2018;23:2347.
11. Sinha N, Yang H, Janse D, et al. Microfluidic chip for precise trapping of single cells and temporal analysis of signaling dynamics. *Commun Eng* 2022;1:18.
12. Morata-Tarifa C, Jiménez G, García MA, et al. Low adherent cancer cell subpopulations are enriched in tumorigenic and metastatic epithelial-to-mesenchymal transition-induced cancer stem-like cells. *Sci Rep* 2016;6:18772.
13. Shih YH, Chen CC, Kuo YH, et al. Caffeic acid phenethyl ester and caffeamide derivatives suppress oral squamous cell carcinoma cells. *Int J Mol Sci* 2023;24:9819.
14. Ohnishi Y, Minamino Y, Kakudo K, Nozaki M. Resistance of oral squamous cell carcinoma cells to cetuximab is associated with EGFR insensitivity and enhanced stem cell-like potency. *Oncol Rep* 2014;32:780—6.
15. Singh P, Augustine D, Rao RS, et al. Role of cancer stem cells in head-and-neck squamous cell carcinoma - a systematic review. *J Carcinog* 2021;20:12.
16. Yue H, Hu Z, Hu R, et al. ALDH1A1 in cancers: bidirectional function, drug resistance, and regulatory mechanism. *Front Oncol* 2022;12:918778.
17. Gupta V, Maurya MK, Agarwal P, et al. Expression of aldehyde dehydrogenase 1A1 in oral squamous cell carcinoma and its correlation with clinicopathological parameters. *Natl J Maxillofac Surg* 2022;13:208—15.
18. Yu CC, Hu FW, Yu CH, Chou MY. Targeting CD133 in the enhancement of chemosensitivity in oral squamous cell carcinoma-derived side population cancer stem cells. *Head Neck* 2016;38(Suppl 1):E231—8.
19. Senbanjo LT, Chellaiah MA. CD44: a multifunctional cell surface adhesion receptor is a regulator of progression and metastasis of cancer cells. *Front Cell Dev Biol* 2017;5:18.
20. Smith SM, Cai L. Cell specific CD44 expression in breast cancer requires the interaction of AP-1 and NFκB with a novel cis-element. *PLoS One* 2012;7:e50867.
21. Judd NP, Winkler AE, Murillo-Sauca O, et al. ERK1/2 regulation of CD44 modulates oral cancer aggressiveness. *Cancer Res* 2012;72:365—74.
22. Mani SA, Guo W, Liao MJ, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* 2008;133:704—15.
23. Miyazaki H, Takahashi RU, Prieto-Vila M, et al. CD44 exerts a functional role during EMT induction in cisplatin-resistant head and neck cancer cells. *Oncotarget* 2018;9:10029—41.
24. Omrani V, Targhi MZ, Rahbarizadeh F, Nosrati R. High-throughput isolation of cancer cells in spiral microchannel by changing the direction, magnitude and location of the maximum velocity. *Sci Rep* 2023;13:3213.
25. Liang F, Peng J, Shi J, Wang L, Aimé C, Chen Y. Microfluidic release of the rare cells captured by a filter with tapered holes. *Micro and Nano Engineering* 2022;14:100119.
26. Macaraniag C, Luan Q, Zhou J, Papautsky I. Microfluidic techniques for isolation, formation, and characterization of circulating tumor cells and clusters. *APL Bioeng* 2022;6:031501.
27. Masuda T, Song W, Nakanishi H, Lei W, Noor AM, Arai F. Rare cell isolation and recovery on open-channel microfluidic chip. *PLoS One* 2017;12:e0174937.
28. Vaccariello M, Javaherian A, Wang Y, Fusenig NE, Garlick JA. Cell interactions control the fate of malignant keratinocytes in an organotypic model of early neoplasia. *J Invest Dermatol* 1999;113:384—91.
29. Braakhuis BJM, Leemans CR, Brakenhoff RH. Expanding fields of genetically altered cells in head and neck squamous carcinogenesis. *Semin Cancer Biol* 2005;15:113—20.
30. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990;61:759—67.
31. Fong EJ, Huang C, Hamilton J, et al. A microfluidic platform for precision small-volume sample processing and its use to size separate biological particles with an acoustic microdevice. *J Vis Exp* 2015;(105):53051.
32. Greenfield EA. Single-cell cloning of hybridoma cells by limiting dilution. *Cold Spring Harb Protoc* 2019;2019:726—8.
33. Mathupala S, Sloan AA. An agarose-based cloning-ring anchoring method for isolation of viable cell clones. *Biotechniques* 2009;46:305—7.