Cancer Stem Cell Traits in Tumor Spheres Derived from Primary Laryngeal Carcinoma Cell Lines

Abstract

Objective: Cancer stem cells (CSCs) belong to a subpopulation of undifferentiated cells present within tumors that have the potential to regenerate, differentiate, maintenance of pluripotency, drug resistance, and tumorigenicity when transplanted into an innate host. These can influence the growth and behavior of these tumors and are used to investigate the initiation, progression, and treatment strategies of laryngeal cancer. Research on CSC science and targeted therapies were hinge on their isolation and/or enrichment procedures. The object of the study is to isolate cancer stem cells from primary laryngeal carcinoma (CSCPLC) by tumor spheres enrichment. We checked the properties of self-renewal, stemness, clonogenicity, and chemotherapeutic resistance. **Materials and Methods:** We performed tumor sphere formation assay (primary, secondary, and tertiary) chemotherapy resistance by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay were performed to evaluate the CSC cells. Immunofluorescence for stem cell markers (CD133+, CD44+) and gene expression of stem cell markers for CD133+, CD44+, OCT4, SOX2, and NANOG was done using the real-time polymerase chain reaction technique. **Results:** We were able to isolated CSC subpopulations from PLC cell lines by the tumor sphere method. These cells exhibited good primary, secondary, and tertiary tumor sphere formation efficiency and also disclosed a resistant index of more than 2. Immunofluorescence for stem cell markers (CD133+ and CD44+) confirms the presence of CSC. There was significantly higher mRNA expression of stem cell markers in CSC enriched subpopulations compared to the parental cell lines. **Conclusion:** We conclude that tumor spheres enrichment is an efficient, economical, and reliable approach for the isolation and characterization of CSC from PLC cell lines. These cells demonstrated the properties of self-renewal, stemness, clonogenicity, and chemotherapeutic resistance.

Keywords: *Cancer stem cells, chemoresistant, immunofluorescence, tumor spheres*

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Submitted: 20-Apr-2020
Revised: 21-May-2020
Accepted: 19-Jul-2020
Published: 21-Sep-2021

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Access this article online

Website: www.contempclindent.org

DOI: 10.4103/ccd.ccd_252_20

Quick Response Code:

Introduction

Among head and neck cancer, laryngeal cancer is considered the second most common cancer and accounts for 2.4% of all cancer cases and 2.1% of all cancer deaths worldwide.[1] Approximately 1,59,000 new cases are being diagnosed and 90,000 cancer deaths occur annually worldwide. The incidence rates of laryngeal cancer were reported previously in a range of 1.26–8.18/100,000 population of India and are known to be the most common cause of death in men.[2] Regardless of the recent developments in surgical expertise and adjuvant treatments, the overall prognosis of laryngeal carcinoma remains unaffected. However, treatments such as tumor resection, chemotherapy, radiotherapy, simultaneous chemo-radiotherapy, and gene therapy have reported a better prognosis with a 5-year survival rate; still, 30%–40% of patients die due to local invasion, recurrence, or metastasis.[3] This is due to the lack of knowledge on its pathogenesis, invasion, and metastasis which needs to be understood to develop new treatment approaches.[4,5] Conventional therapies have been designed to target the bulk of a tumor with the assumption that all cells within a cancer are equally tumorigenic. Nevertheless, recent studies found that each tumor is organized in a hierarchy of heterogeneous cell populations and contains a small, therapy-resistant sub-population of cells – the cancer stem cells (CSCs) – displaying tissue-specific stem cell properties, responsible for tumor initiation, propagation, and regeneration. Hence, the detection and eradication of these CSCs have been considered essential for cancer treatment.[6]

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How to cite this article: Kumbar VM, Muddapur UM, Bhat KG, Shwetha HR, Kugaji MS, Peram MR, *et al.* Cancer stem cell traits in tumor spheres derived from primary laryngeal carcinoma cell lines. Contemp Clin Dent 2021;12:247-54.

Fluorescence-activated cell sorting analysis and magnetic-activated cell sorting are some of the methods to isolate and identify the CSCs population. These are a robust tool, very expensive with a high-maintenance cost along with several drawbacks such as machine calibration, use of proper controls, and compensation-gating protocols.[7] The economical method to isolate CSC includes culturing cells in ultra-low attachment plates in serum-free conditions supplanted with specific mitogens, such as epithelial growth factor (EGF) and basic fibroblast growth factor (FGF).[8] Previously anchorage-independent method was effectively used for propagating breast CSCs. In such an environment, these cells grew as three-dimensional multicellular clones called "Tumor spheres."[9] Tumor spheres or spheroid enrichment technique is an efficient, economical, and reliable approach for studying CSC, including the examination of therapeutic response. Spheroid enrichment conditions have been standardized, facilitating reproducibility.[10] Many cell line studies (breast, liver, colon, and ovarian) showed that enrichment of CSC can be done by the tumorspheres culture method.[11] Recently, the spheroid enrichment method was used to isolate the CSC from three glioma cell lines (LI, U87, and U373) and also from oral squamous carcinoma cell lines.[12,13]

There are many markers studied to identify these CSCs, few of them being aldehyde dehydrogenase-1, CD44, CD24, and CD133 which are most commonly used for solid tumors.[14] CD44 plays a key role for the cell to cell and cell to matrix interactions, and it also helps the growth factors to accumulate on cell surface along with cell adhesion. CD44 expression in head and neck cancers, including primary laryngeal carcinoma (PLC), was allied with poor prognosis. CD133, a transmembrane glycoprotein expressed in endothelial progenitor cells, hematopoietic stem cells, and it is a defined marker for PLC and several other tumors.[15]

In our current study, we sought to isolate the CSCs from PLC by tumorspheres enrichment method and resistance to chemotherapy. We also confirmed the expression of stem cell markers CD133 + and CD44 + by immunofluorescence. Furthermore, the gene expression of stem cell markers, including CD133, CD44, OCT4, SOX2, and NANOG, were carried out by using the real-time polymerase chain reaction (RT-PCR) technique.

Materials and Methods

Primary culture

The study was approved by the ethics committee of our institution (No-1452). Human laryngeal carcinomas tissue specimens were obtained during the standard surgical procedure after taking consent from five patients (PLC 1–5). Tissue specimens were transported to the laboratory by phosphate buffer solution and processed immediately for culture by enzymatic technique. Primary tumor samples were rinsed in Dulbecco's modified Eagle's medium (DMEM)/F12 medium with gentamicin (50 µg/ml) and amphotericin B (50 µg/ml) to minimize the risk of microbial contamination. After rinsing with a 10% povidone-iodine solution for 1 min, immediately wash with Phosphate buffer saline (PBS) thrice and twice with culture media. Then, tissues were carefully minced into the small pieces of approximately 2 mm in size using a sterile razor blade and placed in a 15 ml centrifuge tube containing dissociation solution (DMEM-F12 with 300 U/mL collagenase IV, 100U/mL hyaluronidase, and 125 U/mL DNAse). Tubes were incubated at 37°C for 30 min. Pipette up and down with a 1 mL pipette to dissociate cells every 10–15 min until the tumor is well dissociated. Fibroblasts were removed by brief exposure to 0.25% trypsin-EDTA, and the primary cancer cell cultures were obtained after the continuous successful passage of laryngeal squamous cells. Thus, the fibroblasts were eliminated. The culture plates were examined daily by an inverted microscope for the growth and any contamination. The old culture medium was replaced with a fresh one twice a week.[16]

Tumorsphere culture

Approximately 5×10^3 viable cells were seeded in a 12-well ultra-low attachment plate. Then 200 µl of serum-free DMEM/F12 medium supplement with, FGF (20 ng/mL), EGF (20 ng/mL), N_2 supplement was added and maintained in a CO_2 incubator (Galaxy 170R, Eppendorf, New Brunswick, Germany) at 37°C, 5% CO_2 with 95% humidity. The media was changed for every 3rd day. Once the spheres were formed by about 14–20 days, they were collected in a 15 ml centrifuge tube with the help of micropipettes and centrifuge at 1300 rpm for 3 min followed by washing with PBS. 1 ml 0.025% trypsin/EDTA was added and gentle pipetting done for 3 min until the spheres were broke to form unicellular cell suspension.[17]

Tumorsphere forming efficiency assay

The cell suspension was diluted to get one cell per 100 µl tumorsphere culture media. Then, 100 µl of this suspension was added to each of 96-well ultra-low attachment plates. Wells containing two or more cells were marked and were not used subsequently. Sphere formation was scored after 2 weeks of incubation and percentage of cells that formed sphere was determined by the following formula: $Y(n)/X(n) \times 100$.

Where $X(n)$ is the number of wells in which a single cell was present

$Y(n)$ is the number of wells in which tumorsphere formed from a single cell.

The unicellular cell suspension was prepared by trypsinization of the formed tumorsphere. Then 100 µl tumorsphere culture media was added into each well of 96-well ultra-low attachment plate and checked for

secondary sphere formation efficacy.[12] After the formation of a single-cell colony, these cells were seeded into 6-well plates for further analysis.

Chemotherapy resistance assay

We performed chemotherapy resistance of CSCs by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Approximately 5×10^3 cells were seeded in a 96-well flat-bottomed polystyrene microtiter plate (NEST-Biotechnology) and maintained at 37°C in 95% humidity and 5% CO_2 overnight. The cells were treated with different concentrations (25, 20, 15, 10, 5, 1 µM/mL) of cisplatin and incubated for 48 h followed by washing wells twice with PBS. 20 µl of the MTT reagent solution (5 mg/ml in PBS) was added to each well and the plate was incubated for 4 h in CO_2 incubator. After incubation, 100 µL of dimethyl sulfoxide was added to each well to dissolve the formazan crystals. The absorbance was recorded at 570 nm using a microplate reader (Bio-Rad, California, USA).[18]

Formula: Surviving cells (%) = mean optical density (OD) of test compound/mean OD of negative control ×100.

Resistant index (RI) more or equal to 2 considered as chemoresistant.

Immunofluorescence

The cells which formed single-cell colony were seeded at a density of approximately 2×10^4 cells in each well-containing coverslips in a 24-well flat-bottom microplate and maintained at 37°C in 95% humidity and 5% CO_2 for overnight. After overnight incubation, the cells were fixed for 30 min in 4% paraformaldehyde. The cells were then washed with a phosphate-buffered solution and incubated with a blocking solution containing PBS with 1% bovine serum albumin, 0.1% Triton X-100 for 30 min. Again cells were washed with PBS and treated with secondary antibodies for 1 h. The cells were then washed three times in PBS and examined under a fluorescent microscope (Olympus BX41) at ×20 magnification.[19]

Real time-polymerase chain reaction

Total RNA was extracted using Qiazol reagent (Qiagen, Hilden, Germany). The RNA pellet was dissolved in RNAse free water. The cDNA conversion was carried out by using the PrimeScript RT reagent kit (Takara, Shiga Prefecture, Japan). The sequences of specific primers used are shown in Table 1. The reaction mixture was prepared in a total volume of 20 µl and PCR thermal cycling conditions were performed in Realplex master cycler (Eppendorf, Germany). Initial denaturation was done at 95°C, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and 72°C for 20 s. Melting curve analysis was performed to confirm the specificity of primers. Cycle thresholds (ct value) for all the samples were obtained. The ratios of specific mRNA expressions were normalized by the value of the housekeeping gene with β-actin.[20]

Statistical analysis

All experiments were performed in triplicate, and the results were expressed as a mean and standard deviation. Statistical analysis was carried out using GraphPad Software Inc., CA, USA with one-way analysis of variance followed by Tukey's multiple comparison tests. The difference was regarded as statistically significant when $*P < 0.05$ (Significant), $**P < 0.01$ (moderately significant), and $***P < 0.001$ (highly significant).

Results

Sphere forming assay

Sphere formation has been used to isolate and assess the characteristics of CSCs from the PLCs. Figure 1a clearly showed that these cells gradually started to detach and started sphere formations in DMEM/F12 serum-free medium with basic FGF and EGF. After the completion of 2 weeks of incubation, these cells progressively formed spherical colonies of various sizes and shapes in all samples, as shown in Figure 1b.

Tumor sphere forming efficiency

Monoclonal subpopulations of CSCs from the single-cell were enriched through sphere formation, and it was used to evaluate the tumor sphere-forming efficacy. Figure 2a

Table 1: Primers used for the real-time polymerase chain reaction analysis

Target genes	Sequences
NANOG F	TGAGATGCCTCACACGGAGACTG
NANOG R	GGGTTGTTTGCCTTTGGGACTG
OCT3/4 F	GGTGCCTGCCCTTCTAGGAATG
OCT3/4 R	TGCCCCCACCCTTTGTGTTC
SOX2F	CAACGGCAGCTACAGCATGATG
SOX2 R	GCGAGCTGGTCATGGAGTTGTACT
CD133 F	TGCTGCTTGTGGAATAGACAGAATG
CD133 R	AGGAAGGACTCGTTGCTGGTGAA
CD44 F	CGGACACCATGGACAAGTTT
CD44 R	GAAAGCCTTGCAGAGGTCAG

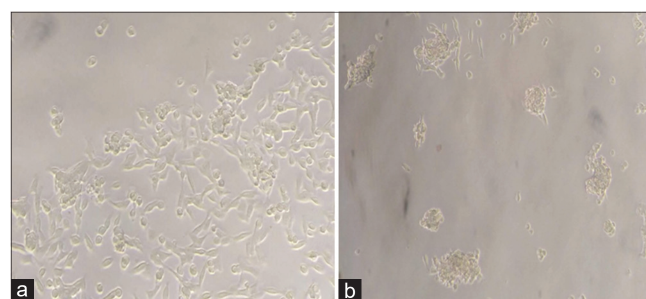

Figure 1: Microscopic pictures presenting the morphology of tumor sphere (a) cells gradually started to detach and started sphere formations in Dulbecco's modified Eagle's medium/F12 serum-free medium with basic fibroblast growth factor and epithelial growth factor (b) after completion of 14 days incubation, spherical sized colonies of various sizes of tumorspheres

shows sphere formations from the single-cell after 3 weeks of culture and an increase in the volume of the sphere-like body with long time cultivation. The secondary and tertiary tumorsphere formation was also shown in Figure 2b and c. The primary, secondary, and tertiary tumorsphere formation efficiency of CSCPLC 1–4 was in the range of 50.52–19.87, as shown in Table 2. There was a decrease in tumorsphere formation from primary to tertiary, but these differences were not statistically significant. The primary tumorsphere formation efficiency of CSCPLC 5 subpopulation was 29.05 ± 4.94, whereas no formation of secondary and tertiary tumorsphere was observed.

Chemotherapy resistance assay

The chemoresistance of isolated CSC lines was done by cisplatin drug. RI more or equal to 2 was considered to show chemoresistant property. An isolated subpopulation of CSC of PLC (CSCPLC 1–3) RI was almost 3. Whereas RI for CSCPLC4 and CSCPLC5 were just above 2 as shown in Table 3.

PLC1–3 was showing more significant cell death at <5 μM/ml concentrations of cisplatin treatment in comparison with subpopulations CSC of PLC (CSCPLC 1–3). At higher concentrations, i.e., 15 μM/ml concentrations of cisplatin treatment showed significant cell death when compared PLC4–5 with subpopulations CSC of PLC (CSCPLC 4–5), as shown in Figure 3.

Immunofluorescence

The FITC-conjugated CD133 antibody (green) and Allophycocyanin (APC)-conjugated CD44 antibody (red) were used for the confirmation of the presence of CSC by immunofluorescence. The counter-stain DAPI was used to stain the nuclei. The fluorescent distribution pattern of immunostaining confirmed the existence of CD44+ and CD133+ cells is shown in Figure 4.

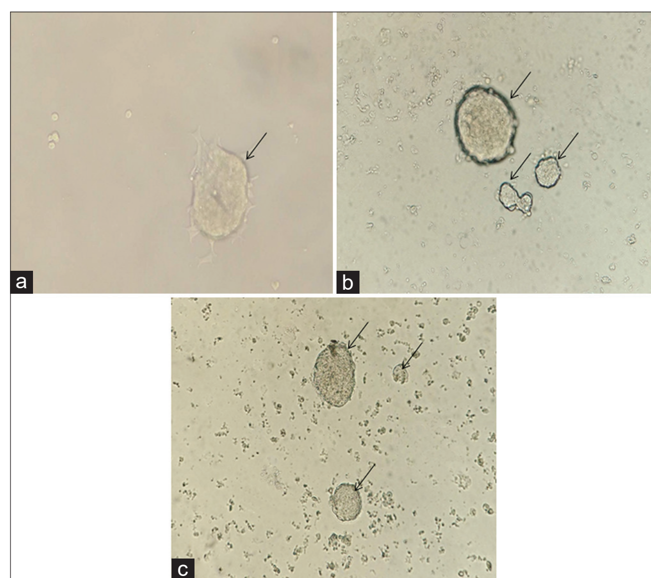

Figure 2: Tumorsphere forming efficiency (a) primary sphere formation (b) secondary sphere formation and (c) tertiary sphere formation

Real time-polymerase chain reaction

Real-time PCR analysis revealed that the mRNA expression levels of some stem cell markers including CD133, CD44, OCT4, SOX2, and NANOG were significantly higher in CSC-enriched subpopulations compared to the parental cell lines, as shown in Figure 5.

Discussion

The larynx is the second most common site of head and neck squamous carcinomas. Laryngeal cancer is known to have properties of local invasion, regional metastasis of lymph nodes, and chemoresistance leading to poor prognosis with a high rate of morbidity and mortality worldwide including India. The 5-year survival for laryngeal cancer in India accounts for approximately 28%.[21] This low survival rate is due to the presence of CSCs which are responsible for local recurrence, distant metastasis, and therapeutic resistance.[22] A meta-analysis study on CSC markers by Fan *et al*. was suggestively related with poor overall survival and disease free survival but not disease specific survival of squamous cell carcinoma patients. Nevertheless, there were different subgroups which showed varying results, suggesting the future large sample size longitudinal studies to confirm the findings.[23] Recent studies showed

Table 2: Tumor sphere forming efficiency (%)

Cell populations	Tumor sphere formation efficiency (%)		
	Primary	Secondary	Tertiary
CSCPLC1	40.62±6.07	36.14±5.28	34±4.63
CSCPLC2	50.52±4.36	44.13±4.82	41.05±5.05
CSCPLC3	36.10±5.01	43.25±4.29	38.55±5.79
CSCPLC4	35.77±4.22	24.94±6.06	19.87±4.94
CSCPLC5	29.05±4.94	-	-

CSCPLC1-5: Cancer stem cell population isolated from primary laryngeal carcinoma of five patients

Table 3: Half maximal inhibitory concentration values of *cisplatin* on primary and cancer stem cell populations

Cell line	Cis-platin (IC_{50} in μM/ml)	RI
PLC1	13.74	-
CSCPLC1	40.46	2.944687
PLC2	14.72	-
CSCPLC2	45.19	3.069973
PLC3	14.17	-
CSCPLC3	41.94	2.959774
PLC4	18.26	-
CSCPLC4	37.64	2.0613362
PLC5	17.33	-
CSCPLC5	30.21	1.74322

Resistant index of cancer stem cell populations. PCL: Primary laryngeal carcinoma; CSCPLC1-5: Cancer stem cell population isolated from primary laryngeal carcinoma of five patients; RI: Resistant index; IC_{50}: Half maximal inhibitory concentration

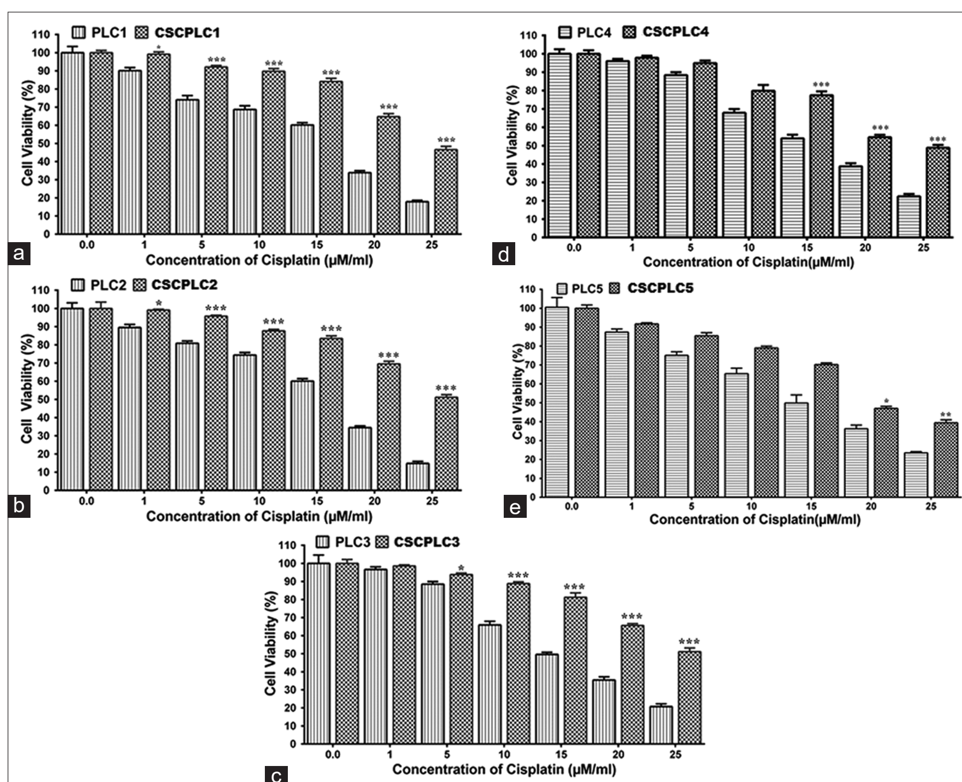

Figure 3: Chemotherapy resistance assay-Graph showing the cell viability of primary laryngeal carcinoma 1–5 and CSCPLC 1–5 (a, b, c, d, and e) population after treatment with cisplatin. Data are mean ± standard deviation of thee independent experiments. Significant difference specified as ***P < 0.001, **P < 0.01, *P < 0.05 and ns-not significant between control versus treated samples. Primary laryngeal carcinoma 1–5 denotes to primary laryngeal carcinoma obtained from five patients. CSCPLC1–5 denotes to Cancer stem cell population isolated from primary laryngeal carcinoma. CSCPLC: Cancer stem cells from primary laryngeal carcinoma

that epithelial-mesenchymal transition (EMT) is implicated in the attainment of CSC properties. Cells within spheroids showed higher expression reveal of the stemness-related transcription factors Oct3/4, SOX2 and NANOG, upregulation of Snail, Twist, alpha-smooth muscle actin and Vimentin, and downregulation of E-cadherin. The upregulation of EMT/CSC pathways can have a bearing on patient treatment as it has been associated with poor prognosis and resistance to radiotherapy and chemotherapy.[24] It has been established that CSCs also interact with the cellular components of tumor cells and their microenvironment to form the metastasis cascade, by establishing premetastatic niche for their onset through cellular and molecular mechanisms.[22] The isolation and culture of CSC is a key step to explore the chemoresistance mechanism of CSC. Tumor sphere formation assay was most commonly used to study the stemness, self-renewal, and clonogenicity of CSC.[23] First time implemented the model to culture the neural stem cells as neurospheres.[24] After this innovation, everybody started culturing cells in nonadherent and serum-free conditions to enrich the stemness property of cells. Spheroid enrichment is a resourceful, cost-effective, and reliable approach with specific culture conditions that have been applied for facilitating standardized, reproducibility results.[10] Pozzi *et al*. showed that modified culture conditions such as serum-free media with specific growth factors in low attachment culture plates were used for the CSC by the tumorspheres enrichment method.[20] Harper *et al*. in his study revealed that holoclone morphology colonies contain undifferentiated, stem cell properties in head and neck squamous cell carcinoma (HNSCC).[19] Felthaus *et al*. study confirmed that single CSCs of established oral squamous cell carcinoma (OSCC) cell lines showed holoclone colonies formation with the positive expression for stem cell markers CD133, CD44, OCT4, SOX2, NANOG, and Nestin.[13] In our present study, we used the serum-free media with human recombinant EGF and basic FGF for tumorsphere formation assay. The tumorsphere formation occurs after 3 weeks of incubation. EGF binds to its receptors results in dimerization and phosphorylation of receptors, thus activating signaling cascade such as MAPK, AKT, and JAK-STAT which are responsible for self-renewal of cancer cells. Similarly, it promotes the transcription factors, namely Notch, Shh, Oct3/4, and Wnt which are involved in the maintenance of pluripotency and self-renewal of the stem cell population.[25] The interaction between FGF and its receptors results in the activation of signaling effectors such as MAPK and PI3K/AKT, which are responsible for the self-renewal of cancer cells.[26] Pozzi *et al*. showed that modified culture conditions such as serum-free media with growth factors showed sphere

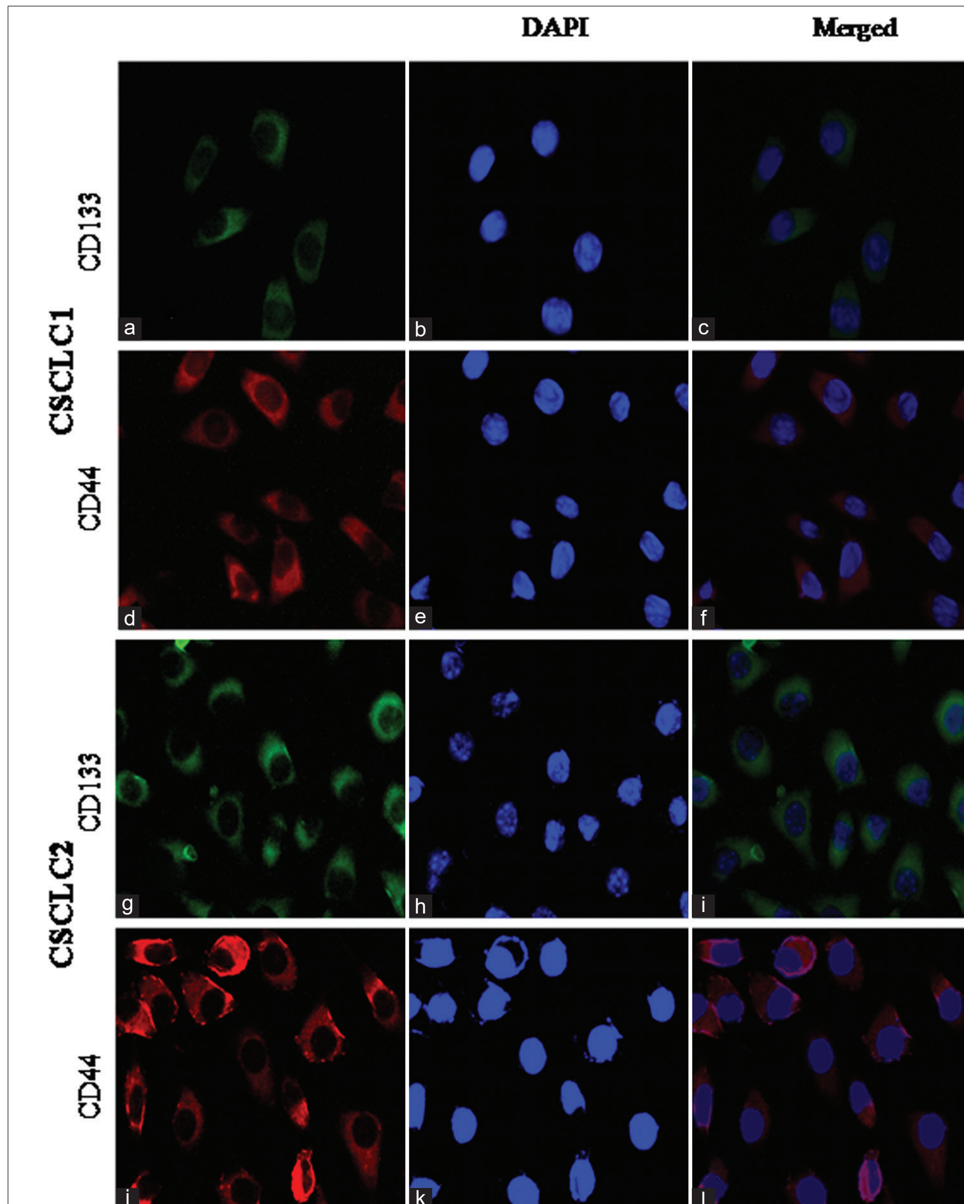

Figure 4: Immunofluorescence; Immunofluorescent staining of cancer stem cells populations – (a) and (g) are FITC-conjugated CD133 antibody (green), (d) and (j) are Allophycocyanin-conjugated CD44 antibody (red) markers, (b), (e), (h) and (k) are DAPI (Blue) stained and (c), (f), (i) and (l) are merged

formation occurs after 3 weeks of incubation of enrichment CSC subpopulation isolated from the HEP2 cell line and primary HNSCC culture, whereas parental cell lines did not show any sphere formation.[20] Calvet *et al*. study showed that sphere formation occurs in murine melanoma cancer cell line (B16-F10), human colon adenocarcinoma (HT-29), and human breast adenocarcinoma (MCF-7) cell lines, whereas MDA-MB-231 cell line did not show any sphere formation due its high passage number.[17]

The primary, secondary, and tertiary tumorsphere formation efficiency of subpopulations CSC of PLC (CSCPLC 1–3) was above 35% after few passages confirming the self-renewal property which is a trademark of CSC. Whereas primary, secondary, and tertiary tumorsphere formation efficiency as of CSCPLC-4 was gradual decreases and in case of CSCPLC-5, there no secondary and tertiary tumorsphere formation occurred. This could be due to more number of cell passages which will affect the tumorsphere formation.[17] Chemo-resistance is one of the unique properties of CSC. It has been extensively defined that CSCs have additional advanced resistance mechanisms for chemotherapy in contradiction to non-CSC and differentiated tumor cells.[27] RI more or equal to 2 considered as chemo-resistance. RI of subpopulations CSC of PLCs (CSCPLC 1–3) was more than 2. CD133+ and CD44+ cells showed significantly highest cell viability after treatment of cisplatin at 0, 1, 5, 10, 15, 20, and 25 μM/ml for 48 h in comparison with parental cells. CD133, CD44, and ALDH1 are the most common markers that were used to identification and isolation of CSC in

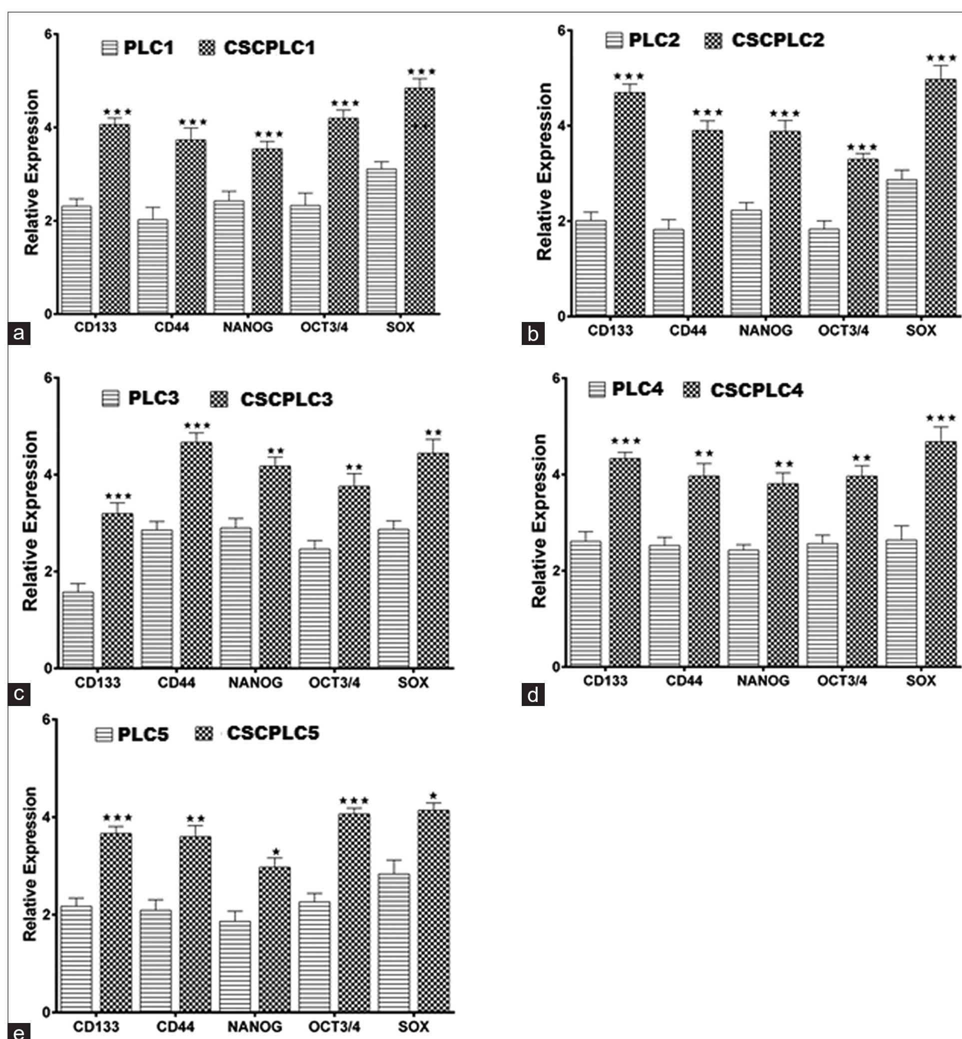

Figure 5: Stem cell markers CD133, CD44, OCT4, SOX2 and NANOG. Data is mean ± standard deviation of three independent experiments. Significant difference indicated as ***P < 0.001, **P < 0.01, *P < 0.05 between control versus treated samples. Primary laryngeal carcinoma 1–5 denotes to primary laryngeal carcinoma obtained from five patients. CSCPLC1-5 (a, b, c, d, and e) denotes to cancer stem cell population isolated from primary laryngeal carcinoma. CSCPLC: Cancer stem cells from primary laryngeal carcinoma

HNSCC. Many studies were shown that a single surface marker approach failed to produce an optimized method of identification and isolation of CSC in HNSCC.[16] In our study, we used double-positive antibodies FITC-conjugated CD133 antibody and APC-conjugated CD44 antibody for the confirmation of CSC. Kaseb *et al*.'s study showed that staining of the spheroids using stem cell markers (CD44, CD133, SOX2, and BMI1) showed heterogeneous expression.[16] The maintenance of pluripotency and self-renewal of the stem cell population was done by transcription factors OCT4, NANOG, and SOX2 along with other transcription factors (STAT3, HesX1, and Zic3) and cell signaling molecules (TCF3, FGF2, and LEFTY2).[28] Real-time PCR analysis revealed that the mRNA expression levels of some stem cell markers, including CD133, CD44, OCT4, SOX2, and NANOG, were significantly more in CSC-enriched subpopulations when compared with the parental cell lines. Pozzi *et al*. showed that overexpression of OCT4 and NANOG genes found in CSC-enriched subpopulation derived from HNSCC sphere formation colonies, positively correlated with treatment failure, and stage while negatively correlated with differentiation status.[20] Lim *et al*. showed that transcriptional factors (OCT4 and SOX2) were upregulated in spheroid forming cells (i.e., stem-like cells) sorted from human HNSCC.[29] Singh *et al*. confirmed that OCT4, NANOG, and CD133 expression showed the worst survival prognosis in OSCC patients.[30] CD133, CD44, OCT4, SOX2, and NANOG were expressively higher in CSC-enriched subpopulations in comparison with the parental cell lines. Collectively, these data indicate that cells that exhibit stem-like features in cancer express the transcriptional factors OCT4, SOX2, and NANOG. Small size is the limitation of the present study.

Conclusion

In summary, we conclude that the tumorspheres enrichment method may be an efficient, economical, and reliable

approach for the isolation and characterization of CSC from PLC cell lines by considering the cost factor.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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