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Research Article

IDENTIFICATION AND SCREENING OF CANCER STEM CELLS IN ORAL SQUAMOUS CELL CARCINOMA WITH IMMUNOFLUOROSCENCE AND WESTERN BLOT

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ABSTRACT

Background: According to the 'cancer stem cell model' of carcinogenesis, the tumorigenesis is dependent on cancer stem cells (CSCs). Recent reports suggests a key role for these CSCs in prognosis and aids in treatment decision making in various cancers including oral squamous cell carcinomas (OSCCs). Hence, identification of CSCs is of prime importance. Although there are various molecules that serve as markers for these CSCs are available, CD44 and CD 133 are well studied and hence we utilized these markers to identify CSCs in OSCCs.

Methods: Tissue biopsies from 25 OSCC patients and normal individuals were included to study CD44 and CD133 expression by immunofluoroscence and western blot.

Results: In immunofluoroscence, CD44 was expressed in 17/25 OSCCs and 3/25 of normal subjects while CD133 was expressed in 15/25 OSCCs and 3/25 normal subjects. Co-expression of Both proteins was found in 13/25 OSCCs samples, while only one of the normal subject had the co-expression. All the above results were confirmed by western blot. Furthermore, CD44 and CD133 demonstration by western blot revealed varied band intensity which correlated with degree of OSCC differentiation in that darker the band, poor the differentiation.

Conclusion: CD44 and CD133 molecules can be used reliably as CSC markers. Hence, it is of prime importance to isolate, characterize, quantify these CSCs that may aid in identifying patient with poorer prognosis and contribute to therapeutic decisions for OSCCs.

INTRODUCTION

Oral squamous cell carcinoma accounts for more than 100,000 deaths annually. In developing countries, the combination of ageing and exposure to increased levels of cancer risk factors contributes to the rise in cancer and cancer associated deaths.[1] Despite significant advancements in the field of onco-surgery and radiotherapy, there is only a modest improvement in overall survival of these patients.[2,3,4,5] The plausible explanation for this could be that occult tumor cells may remain in the body after treatment and are undetectable by the current diagnostic methods.[6]

Cancer stem cells, a small subset of cells present in cancers constitute a reservoir of self-sustaining cells with the exclusive ability to self-renew and maintain the tumor. These cancer stem cells have the capacity to both divide and differentiate into heterogeneous non-tumorigenic cancer cell types that in most cases appear to constitute the bulk of the cancer.[7] If these cells do not form the targets for currently available treatment modalities then relapses would be expected.[8] Hence current treatment regimens of eliminating high mitotic but terminally differentiated cells of tumors are to be refocused on the targeting these stem cell population.

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Oral squamous cell carcinomas (OSCCs) are one of the aggressive tumors and grow rapidly. The rapid growth of OSCCs points to the presence of stem cells and till date no studies have definitely indicated that stem cells are responsible for oral squamous cell carcinogenesis. Recently, several nuclear and cell-surface marker proteins have been identified for the characterization of these stem cells. The CD44 is a cell-surface glycoprotein involved in cell to cell interactions, cell adhesion and cell migration.[9] It is a hyaluronic acid receptor and has interactions with osteopontin, collagens and matrix metalloproteinases.[10] Recent reports show its higher expression on cancer stem cells than on non-tumorigenic cancer cells. Cancer stem cells expressing CD44 positivity represent less than 10% of the population of tumor cells in primary Head and neck squamous cell carcinoma.[11,12] CD133 a highly conserved antigen homologue of mouse Prominin-1 originally identified in a subpopulation of CD34 positive hematopoietic cells derived from fetal liver and bone marrow, has been used to identify and isolate cancer stem cells from several human cancers.[13,14,15] In hepatocellular carcinomas CD133 positive cancer stem cells were shown to confer chemo-resistance in vitro.[16] However, its biological function remains unknown. In the current study, we used CD 44 and CD 133 molecules as cancer stem markers to identify these cancer stem cells in tissue samples of patients with OSCC and normal controls.

MATERIALS AND METHODS

Selection of cases

Twenty five patients with OSCC and 25 apparently normal persons (controls) were included in the present study from the patients attending the Department of Oral Medicine at Government Dental College and Hospital, Hyderabad. The clinical features of each patient were recorded. Of 25 patients with OSCCs, 17 were males and 8 were female. The diagnosis of OSCC was made clinically and was confirmed by histopathological examination of the biopsy. All patients with histopathologic diagnosis of OSCCs were included in the present study. However, patients with diabetes, hypertension and those under immunosuppressive and corticosteroids treatment were excluded from the study.

Tissue Sampling and Biopsy Diagnosis

After obtaining written informed consent from the patients, two biopsy specimens were removed surgically by incisional biopsy from clinically diagnosed OSCCs. One specimen was washed in saline to remove blood and was fixed in 10% neutral buffered formalin and was sent for histopathologic examination. Of the 25 OSCCs 13 were well differentiated, 9 were moderately differentiated and 3 were poorly differentiated OSCCs respectively. The same sample that was embedded in wax was used for immunofluoroscence studies for the expression of CD44 and CD133. Another biopsy specimen was snap frozen in liquid nitrogen and stored at -80º C until further use for protein extraction and western blot studies. Similarly two biopsy samples collected from each normal subject was evaluated by immunofluoroscence and western blot studies.

Immunofluoroscence analysis

Two sections of five micron thickness were cut from each of representative OSCC and normal subjects' sample, taken on polylysine coated slides and grouped into two groups for CD44 (group 1) and CD133 (group 2) respectively. The following procedure was done for Immunofluoroscence.

All the sections were incubated at 60ºC for 8 hours in an incubator and further deparaffinized by soaking in xylene for 15 min repeated thrice. They were kept in 100% ethanol for 10 min and repeated twice. Then they were soaked in 95% ethanol for 5 min. Further they were rehydrated in double distilled water for 5 min. Later antigen retreival was carried out by microwaving the sections in citrate buffer (10mM citrate buffer: 2.94g of sodium citrate trisodium salt dehydrate to 1 liter of double distilled water) and cooked for 8 min. and repeated thrice. These sections were then washed with phosphate buffer saline (PBS) for 5 min. The non specific binding activity was blocked using diluted goat serum (5% goat serum in PBS = 50 µL of serum in 950µL of PBS) for 1hr at room temperature in a humid chamber. Prediluted mouse anti-CD44 monoclonal antibody (BioGenex) was used on group I sections and kept at 4ºC overnight. Rabbit anti-CD133 polyclonal antibody (Abcam, Cambridge, England) was used on group II sections with 1:200 µL and kept at 4º C overnight. All the sections were washed with PBS for 5 min and repeated three times. All the slides were then transferred to the dark room and further procedure was conducted there. Goat anti-mouse FITC (Fluorescein isothiocyanate) Conjugate (GENEI) was used as secondary antibody on Group I with incubation time of 1 hr. Goat anti-rabbit TRITC (Tetramethyl rhodamine isothiocyanate) conjugate was used as secondary antibody on Group II with incubation time of 1 hr. This was followed by PBS washes three times of 5 mins each. For labeling cell nuclei DAPI (4', 6 diamidino-2-phenylindole) in the dilution of 1:2000 was used for 3 min. and then washed in PBS for 5 min thrice. DAPI produces a blue fluorescence with excitation at about 360 nm and emission at about 460 nm when bound to DNA. There is no emission overlap with Fluorescein and rhodamine. VECTASHIELD Mounting Medium was used and cover slip placed over the stained slides. Finally, these slides were observed in confocal microscope (Olympus FluoViewTM FV1000 Confocal Microscope) and photographs taken. The localization and intensity of the fluorescence noted.

Western blot analysis

Among the many variations that exist in protocols for running western blots, the following procedure was chosen during this experiment with standard Tris- Glycine SDS-PAGE gels and nitrocellulose membranes.

The tissue sample was taken and crushed in mortar and pestle by adding liquid nitrogen and RIPA buffer (10 ml of RIPA and 100 µL 10 X protease cocktail were added) to the tissue sample at the ratio of 1:10. Resultant solution was collected in 2ml eppendorf tubes and sonicated for 45 pulsations. The resultant solution was centrifuged at 12000 rpm for 30 min. The supernatant solution containing proteins was used for protein estimation by western blot.

Lowry method was followed for protein estimation which depended on quantifying the color obtained from the reaction of Folin- Ciocalteu Phenol reagent with the tyrosyl residue of an unknown protein and comparing this color value to the color values derived from the standard curve of a standard protein

BSA (Bovine Serum Albumin). Reagent preparation was done with Solution A containing 1 ml of 2% sodium potassium tartarate solution, 1ml of 1% CuSo4 solution and 100 ml of 2% sodium carbonate in 0.1 N NaOH , while Solution B contained Folins reagent 1N and 1 mg/ml of BSA stock solution.

In preparation of standard curve 20 test tubes were taken, BSA solution was aliquoted in duplicates (10, 20, 30, 40, 50, 60, 70, 80 and 90 µL) and two tubes were left empty to be used as blank tubes. Final volume of each tube was bought to 500 µL with distilled water. Then 5ml of solution A was added into each tube and vortexed immediately. This solution was allowed to stand for 10 min at room temperature. Then 500µL of Folins reagent was added and vortexing immediately. Again, the solution was allowed to stand for 10 min at room temperature. Optical density at 670 nm was checked and a graph was plotted for standard curve.

For protein estimation in the tissue sample 10 µL of the homogenized tissue sample is taken to which 5ml of solution A was added into each tube with immediate vortexing. The solution was allowed to stand for 10 min at room temperature. Then 500 µL of Folins reagent was added to each test tube and immediately vortexed. Again, this solution was allowed to stand for 10 min at room temperature. The absorbent was analyzed at 670 nm of wavelength and compared against standard curve to estimate protein in the sample. Sodium Dodecyl Sulphate (SDS) Gel Electrophoresis of proteins was done to estimate the molecular weight of the polypeptide chain. To pour SDS-Polyacrylamide gel, two glass plates (one notched) with three spacers, clamps were assembled. This assembly was made to stand upright using clamps as supports. Some pre-heated 1% Agarose was poured onto glass plate sandwich edges to seal the glass plates.

Resolving Gels contained 10 ml of 10% Tris HCL of PH 8.8. To make approximately 8 ml of reagent, 2.5 ml of Acrylamide stock was added to 3ml of distilled water, 1.875 ml of Tris-HCL of PH 8.8, 75 µl of 10% SDS, 500 µl of 1.5% Ammonium persulphate and 10 µl of TEMED was added at the end. This acrylamide solution was poured into the gap between the glass plates. Sufficient space was left for the stacking gel. Using a pipette the acrylamide solution was carefully overlaid with isobutanol. This gel was placed in a vertical position at room temperature for 30 min. Polymerization was complete after 30 minutes and then the overlay was poured off and the top of the gel was washed several times with deionized water to remove any unpolymerized acrylamide.

To prepare approximately 3ml of stacking gel (2.5 %) 375 µl of Acrylamide was mixed with 1.695ml of distilled water, 750 µl of Tris -HCL of pH 6.8, 30 µl of 10% SDS, 100 µl of 1.5% Ammonium persulphate and 7.5 µl of TEMED and stirred quickly. Polymerization begins as soon as the TEMED has been added hence the mixture is swirled without delay. Stacking gel solution was poured directly onto the surface of the polymerized resolving gel. Immediately a clean Teflon comb was inserted into the stacking gel solution, being careful to avoid trapping air bubbles. More stacking gel solution was added to fill the spaces of the comb completely. The gel was then placed in a vertical position at room temperature for 30 min. to polymerize. While the stacking gel was polymerizing, the samples in eppendorf tubes were by heated to 100º C for 3 minutes in 1X SDS gel-loading buffer to denature the proteins in a "float" in a waterbath. Samples were layered under buffer on stacking gels. Apparatus was connected and electrophoresed.

After polymerization of stacking gel the Teflon comb is removed carefully. Using a squirt bottle, the wells were washed immediately with deionized water to remove any unpolymerized acrylamide. The gel was mounted in the electrophoresis apparatus. Tris-glycine electrophoresis buffer was added to the top and bottom reservoirs. Air bubbles that became trapped at the bottom of the gel between the glass plates were removed. About 24 µl of each of the samples were loaded in predetermined order into the bottom of the wells. An equal volume of IX SDS gel-loading buffer was loaded into unused wells. The electrophoresis apparatus was attached to electric power supply and a voltage of 100V was applied to the gel. The gel was run until the bromphenol blue reached the bottom of the resolving gel (about 4 hours). Then the power supply was turned off. The glass plates were removed from the electrophoresis apparatus and placed on a paper towel. The plates were pried apart with a spatula. The orientation of the gel was marked by cutting a corner from the bottom of the gel that is closest to the leftmost well (slot 1). This gel was subjected to establish western blot.

Western blotting was done with running buffer using 25mM Tris - HCl of pH 8.3 at room temperature, 192 mM glycine and 0.1ml of 9% w/v SDS. Transfer buffer used was 12mM Tris-HCl at pH 8.3 at room temperature, 96 mM glycine and 20% (w/v) methanol. It was chilled to 40C. For wash solution, 20 mM Tris Buffered Saline, Tris - HCl at pH 7.2 - 7.4 at room temperature and 150 mM NaCl was used. For blocking solution 2% (w/v) fatty acid - free bovine serum albumin (BSA) in Wash Solution was used. Gels were run at 100V constant voltage. Electrophoresis was stopped when the dye fright was about 1 cm from the bottom of the gel. While the gel was running, all fiber pads, filter papers and transfer membrane were soaked in transfer buffer carefully to ensure no bubbles. After electrophoresis, the bottom right corner of the gel was cut off to ensure that the gel is oriented correctly in the transfer apparatus. Gel was kept in transfer buffer for 10 min to equilibrate. The transfer cassette was assembled with negative side to have the gel and the positive side the membrane. The gel and membrane were sandwiched between the filter papers. Transfer was run at a constant voltage giving a starting current of 200 mA and kept for transfer overnight at 25V. After transfer the blot was checked for proper stain using a reversible stain called ponceau s stain (0.1% Ponceau s in 1% acetic acid). Then the membrane was placed in blocking solution on a rocker platform for about an hour at room temperature. It was incubated with CD44 diluted in Blocking Solution at concentration of 2 mg/ml on rocker plate for 1 hour at room temperature. It was washed for 5 times in wash solution (Twice in TBS, Once in TBST and Twice in TBS). It was incubated with secondary antibody conjugate (1 mg/ml for chromagenic detection) in blocking solution for about 1 hr at room temperature on a rocker plate. It was again washed for 5 times in the substitute buffer. Substrate is then added carefully.

RESULTS

The age of the 25 OSCC patients ranged from 32 to 80 years with an average age of 52.8 years. The youngest patient was 32

years old and oldest patient was aged 80 years (Table 1). Majority of these patients were males (17/25; 68%) as compared to females (08/25; 32%) respectively. Twenty five apparently normal persons aged between 22 to 75 years (average age 45 years), served as controls. Out of the 25 controls 68% were males and 32% were females.

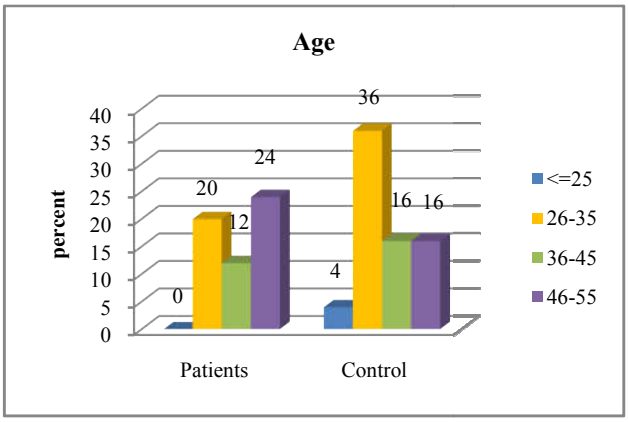

Chart 1 Distribution of age among OSCCs and normal subjects

Twenty one out of 25 OSCC patients presented with non healing ulcers and the rest 4 had exophytic growth. Location wise 17, 5 and, 3 of 25 OSCCs were seen in buccal mucous, alveolus and tongue respectively. Histopathologically, 16, 6 and, 3 OSCC cases exhibited well, moderate and poor differentiation. The expression of CD44, CD133 in the basaloid cells were evaluated and compared with controls using immunofluoroscence and western blot techniques.

CD 44 Expression

Immunofluoroscence

Three of the normal controls (3/25 or 12%) showed CD44 expression, whereas in OSCCs, 17/25 (68%) showed CD44 expression and only 8/25 (32%) did not show CD44 expression. There was a significant variation in the CD44 expression ($P < 0.001$) in OSCCs when compared with normal (Table no. 1) (Chart 2).

Table 1 Expression of CD44 and CD133 among OSCCs and normal subjects by immunofluoroscence and western blot

		Group				P-value
		Cases		Controls		
		N	%	N	%	
CD44	Negative	8	32.0%	22	88.0%	<0.001; Sig
	Positive	17	68.0%	3	12.0%	
CD133	Negative	10	40.0%	21	84.0%	0.001; Sig
	Positive	15	60.0%	4	16.0%	

Chi-square test

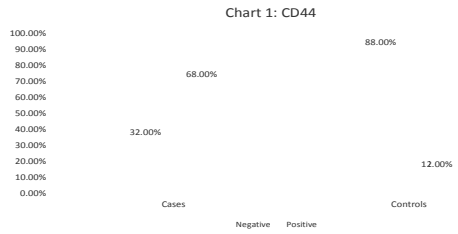

Chart 2 Expression of CD44 among OSCCs and normal subjects by immunofluoroscence and western blot

Western blot technique

The results yielded for CD44 protein expression by western blot also corresponded to the CD44 expression by immunofluoroscence. Out of 17 patients who showed band formation for CD 44 protein, 64% (11/17) showed light band appearance, 24 % (4/17) showed dark band appearance, 12 % (2/17) showed very dark band appearance. On the whole 17/25 (68%) showed band formation and 8/25 (32%) showed no band for the CD44 protein. The band formation by CD44 protein was more intense in the subjects with poorly differentiated oral squamous cell carcinoma compared to the band intensity of CD 44 protein to the moderately differentiated oral squamous cell carcinoma. The CD 44 protein band formed by well differentiated oral squamous cell carcinoma was less intense than the CD 44 protein band formed by moderately differentiated oral squamous cell carcinoma while only light bands were formed in three of the controls. Only one sample of three poorly differentiated OSCC did not show any band formation for CD44, while 2/6 moderately differentiated OSCC samples and 5 /16 well differentiated OSCC did not show band formation for CD44. The band intensity in the 3 of the normal subjects was light.

CD 133 Expression

Immunofluoroscence

16 % (4/25) normal controls had CD133 expression. However 60% (15/25) of OSCCs showed expression of CD133 and 40% (10/25) of OSCCs did not show any CD133 expression. The CD133 expression in OSCCs as compared to normal subjects was statistically significant (P= 0.001) (Table no. 1) (Chart 3).

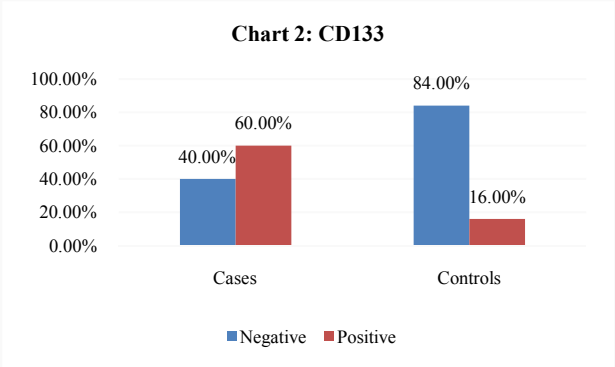

Chart 3 Expression of CD133 among OSCCs and normal subjects by immunofluoroscence and western blot

Western blot technique

In OSCCs the CD133 protein expression by western blot also corresponded exactly to the CD133 expression by immunofluoroscence. Out of 15 patients who showed band formation for CD133 protein, 60% (9/15) showed light band appearance, 27 % (4/15) showed dark band appearance, 13 % (2/15) showed very dark band appearance. On the whole 15/25 (60%) showed band formation and 10/25 (40%) showed no band for the CD133 protein. The band formation by CD133 protein was more intense in the subjects with poorly differentiated oral squamous cell carcinoma compared to the band intensity of CD 133 protein to the moderately differentiated oral squamous cell carcinoma. The CD133 protein band formed by well differentiated oral squamous cell

carcinoma was less intense than the CD133 protein band formed by moderately differentiated oral squamous cell carcinoma. 8 out of 16 well differentiated OSCC did not show any band for CD133, while two of the six moderately differentiated OSCC did not show any band for CD133. Only light band was formed in four of the controls.

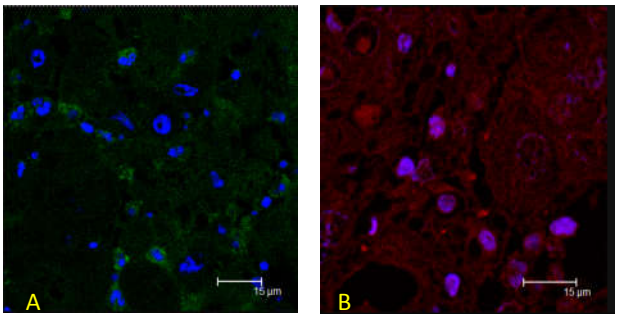

Figure 1 Expression of (A) CD44 (Fluorescein Isothiocyanate) and (B) CD133 (Tetramethyl Rhodamine Isothiocyanate) in OSCCs. Immunofluorosence.

Thirteen out of twenty-five OSCC co-expressed for both CD44 and CD133, while only one of the normal control had co-expression of both CD 44 and CD133 in both fluorescence and western blot analysis. Six of the twenty-five OSCCs and nineteen of the twenty-five normal controls did not show any expression for both CD44 and CD133. Only two in both OSCCs and Normal controls showed expression for CD44 but did not show any expression for CD 133. While two of the OSCCs and three of the normal controls did not show expression for CD44 but showed expression for CD133.

DISCUSSION

Oral Squamous cell carcinoma (OSCC) represents more than 90% of all oral cancers and most of the cases occur in individuals over 40 years of age.[1] Tobacco is the main etiological agent in OSCC followed by viruses. [17] In the present study out of 25 oral squamous cell carcinoma patients the age of the subjects varied from 32 to 80 years with majority of them above 45 years of age as reported by the earlier studies[4, 18, 19] which stated that oral squamous cell carcinoma is a disease of elderly. Oral cancer occurs in men twice as common as in women.[4,18] In the current study 17(68 %) OSCCs were male and 8 (32 %) were female patients with a male to female ratio of more than 2:1 as reported in the earlier studies[4, 19,20,]

Buccal mucosa is the commonest site of involvement by OSCC in the oral cavity in India as compared to the western world where lip, tongue and floor of the mouth are more commonly involved than buccal mucosa.[21] High prevalence of chewing oral tobacco products is presumed to be the major contributor for buccal involvement. Our study also showed buccal mucosa as the most commonly involved site by OSCC in the oral cavity. Alveolus and tongue were the other sites involved by OSCC after buccal mucosa.

Clinically majority of the patients presented with non healing ulcers and only small percentage of patients had exophytic growth. OSCCs are thought to arise from keratinizing or malphigian epithelial cells and the histopathologic hallmark of SCC is the presence of keratin with/without formation of "keratin pearls".[92] According to Andisheh-Tadbir *et al* the most common histological type of OSCC is well differentiated (60%), followed by moderately differentiated (28.5 %) and the poorly differentiated variant being least common (11.5%).[22] In consensus with the above study the current study also demonstrated well differentiated in 64%, moderately differentiated in 24 % and poorly differentiated in 12% of cases respectively.

Even though the concept of cancers arising from germ cells existed over 150 years ago, recent advances in stem cell research has rekindled the interest in cancer stem cell hypothesis. Two important related concepts of this hypothesis are that 1) tumors originate in either tissue stem cells or their immediate progeny through deregulation of the normally tightly regulated process of self-renewal 2) tumors contain a cellular subcomponent that retains key stem cell properties. These properties include self-renewal which drives tumorigenesis and differentiation though aberrant that contributes to cellular heterogeneity. [14,24,25,26]

Recent studies[26] in various tumors have supported the cancer stem cell hypothesis. This hypothesis is a game changer for cancer risk assessment, detection, prognosis, prevention and treatment. Presently, the cancer treatments may be more focused on agents that kill differentiated tumor cells while leaving the minority cancer stem cell population. The development of more effective cancer therapies may thus require targeting this important cell population.

Currently, there is a need to develop more markers for the cancer stem cell identification to prove the validity of this hypothesis. Although a universal marker for cancer stem cells has not been identified yet accumulating evidence has shown that a subset of highly tumorigenic cancer stem cells identified in several common types of solid tumors such as neural [27, 10, 28], colon[29,30,31], hepatocellular[32], lung[16], laryngeal[33], and pancreatic cancers[14], commonly express CD133 and CD44[34] cell-surface glycoproteins. It has been reported that CD133+ subpopulations of cells from different types of solid tumors possess self-renewal capacity and they can be clonally expanded and are exclusively tumorigenic and are able to differentiate into the CD133 counterparts[24, 14,25]. In agreement with these earlier reports the present study identified a small subset of CD133+ and CD44+ cells in OSCCs by immunofluoroscence and western blot techniques.

Numerous studies based on immunohisto-chemical analyses of paraffin-embedded or frozen tissue sections using different monoclonal antibodies to CD44 isoforms and molecular biological techniques have provided evidence that in many types of tumours there is overexpression of CD44 isoforms and aberrant processing of immature CD44 transcripts relative to non-neoplastic control tissues suggesting a role of CD44 in tumor development and progression.[35] In the present Immunofluoroscence study for CD 44, 17 subjects out of 25 showed expression of CD 44 + cells. One study demonstrated CD44 positivity in 80-100% of well-differentiated, 60-85% of moderately differentiated and 40-62% of poorly differentiated OSCCs, respectively.[36]

Though CD133 is often used as a CSC marker in many hematopoietic stem cells and solid tumors including OCSCC, there are controversies regarding the role of CD133 in both tumorigenesis and as cancer stem cell marker, showing contradictory reports. Some of these conflicting reports also

state that the CD133− population is in fact more tumorigenic than CD133 + cells.[37] In the present Immunofluoroscence study for CD 133, 15 cases out of 25 showed expression of CD 133 + cells and only 4 of the 25 samples of normal mucosa showed presence CD133+. The current study results are in concordance with the results obtained by Lili Sun *et al*, who reported presence of CD133+ cells in 100 % OSCC cases and found no expression of CD133 in normal mucosa.[38]

Immunofluoroscence studies in 52% (13/25) cases of OSCCs showed co-expression of CD44 and CD133. In a study done at another site, on recurrent colorectal cancers by Tomoyuki Nagata *et al* (2011) about 41% of the cancer samples expressed both CD44 and CD133 and were associated with poorer prognosis.[39] About 24% (6/25) cases showed no expression for both CD44 and CD133. About 16 % (4/25) showed positive only for CD 44 but did not express CD133 while 20% (2/25) showed positive only for CD 133 but did not express CD44.

To confirm the results of Immunofluoroscence tests on CD 44 and CD133 Western blot studies were conducted in the same cases to detect the presence of both CD44 protein and CD133 protein. It was found that 17 out of 25 samples showed the presence of CD44 protein and that 15 out of 25 samples showed the presence of CD 133 protein. It was also found that the intensity of the band formation correlated with the grade of histological diagnosis. No band was formed in 22 of the 25 controls for CD 44 and 21 of the 25 controls did not have band formation for CD133.

This indicates that the intensity of band formation of CD44 and CD133 has some relation to the histological grading and prognosis of OSCCs. Darker bands in western blot studies correlated with poor differentiation of OSCC whereas, lighter bands correlated with well differentiated OSCCs. Hence, low CD44 and CD133 protein expression in OSCCs attribute better prognosis. This may have the therapeutic implication in that these markers could provide targeted therapies using therapeutic antibodies specific for these and thus elimination of these CSCs could possibly be curative.

The reason for the presence of CD44 and CD133 protein the normal mucosal samples maybe due to the presence of epithelial and mesenchymal stem cells in the normal mucosal tissue. CD44 is also found in hematopoietic stem cells, mesenchymal stem cells, and adipose-derived stem cells, while CD133 is also a marker for hematopoietic stem cells, neural and prostate adult stem cell. Though they are expressed on various adult stem cells, their expression is very low in normal tissues. The western blot demonstrated light bands for the CD44 and CD133 expression in normal oral mucosa samples corroborating with the expression by immunofluoroscence.[40]

SUMMARY AND CONCLUSION

Cancer stem cells (CSCs) are a small sub-population of cancer cells possessing characteristics normally associated with stem cells such as self-renewal and the ability to differentiate into multiple cell types. These cells are tumor-initiating cells causing initiation, growth, recurrence and metastasis of solid tumors in contrast to the bulk of cancer cells which are thought to be non-tumorigenic. Such cells persist in tumors as a distinct population and may cause relapse and metastasis by giving rise to new tumors.

The present study is undertaken to identify the cancer stem cells in OSCCs by using CD 44 and CD133 antibodies as a marker. 25 patients of OSCCs and 25 apparently normal persons as controls were included in the present study. To the best of our knowledge, our study is the first to demonstrate the expression of CD44 and CD133 by both immunofluoroscence and Western blot techniques. Among immunofluoroscence and western blot, the latter was found to be more specific and sensitive as it had varying results according to the prognosis of the carcinoma. The cancer stem cell hypothesis posits that cancer stem cells are minority population of self-renewing cancer cells that fuel tumor growth and remain in patients after conventional therapy is completed. Effective tumor eradication will require agents that can target cancer stem cells while sparing normal stem cells. To achieve effective implementation of new therapies, physicians will require methods of determining the type of cancer stem cells present in a given patient's tumor. The identification and isolation of such tumor stem cells is thus important to more completely understand the progression of malignant disease, as well as to the development of improved specific therapies for cancer. The results described so far about the relation between these common CSC markers are still limited and variable. Therefore, further studies are needed to explore the relationship of the common cell surface markers, in particular CD133 and CD44 in solid tumor CSCs especially in cancer stem cells in OSCCs.

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