

Acquisition of Cancer Stem Cell Behaviour Plays a Role in Drug Resistance to Combination Chemotherapy and Prognosis in Head and Neck Cancer

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Abstract

Objective: Head and Neck Squamous Cell Carcinoma (HNSCC) demonstrate an exceptional initial response to induction chemotherapy; nevertheless, loco-regional relapse is widespread and not clearly understood. In this study, we investigated the role of Cancer Stem Cells (CSCs) in mediating chemo resistance using patient cohorts and cell line models.

Methods: Profiling of CSC markers was carried out in primary untreated (Cohort I, N=33) and post treatment recurrent (Cohort II, N=27) HNSCC patients by Quantitative PCR (Q-PCR) and Immunohistochemistry (IHC). The prognostic significance of these markers was assessed by ROC curves and logistic regression analyses. The stem cell related behaviour of the drug resistant TPF cell lines was assessed by the expression of CSC markers and other properties such as self-renewal, migration and tumorigenicity.

Results: Post-treatment recurrent patients showed an over-expression of CSC markers (*CD44*, *ABCG2* and *NOTCH1*) compared to the treatment naïve cohort. Additionally, *CD44* ($p=0.028$) and *ABCG2* ($p=0.019$), in combination, were poor prognosticators (AUC 0.76). The resistant cell lines (Hep-2 TPF and CAL-27 TPF) were further characterized to delineate the role of CSCs in drug resistance. Analogous to the patients, these cells showed an enrichment of CD44+ cells accompanied by an increased spheroid formation ($p<0.005$) and migratory capacity ($p<0.05$). The up regulation of CSC markers (*CD133*, *BMI* and *NOTCH1*) and their resistance-mediating targets such as drug transporters and survival/anti-apoptotic pathways suggested possible causal mechanisms. Furthermore, the higher clonogenic survival in the presence of cisplatin ($p<0.05$) signifying an increased self-renewal capacity with drug resistance. The Hep-2 TPF (10^2 cells) also showed an increased tumorigenicity (2/3; 9.5-fold increase in tumor burden) as compared to the parental (1/3; 6-fold).

Conclusion: Our findings suggest that TPF combination chemotherapy enriches the resident cache of CSCs, ultimately leading to drug resistance. Consequently, in a sub set of patients, these drug resistant CSCs might contribute towards disease relapse/recurrence.

Keywords: Cancer stem cells; Neoadjuvant chemotherapy; Drug resistance; Recurrence; Head and Neck cancer; Prognosis; Cell lines

Abbreviations: Hep-2 P: Hep-2 parental cell line; Hep-2 TPF: Taxol, platinum & 5-Fluorouracil resistant cell line; CAL-27 P: CAL-27 parental cells; CAL-27 TPF: CAL-27 taxol, platinum & 5-Fluorouracil resistant cell line

Introduction

Induction chemotherapy using platinum, 5-fluorouracil (5FU) and docetaxel followed by concurrent chemo-radiation is the standard of care treatment given for locally advanced laryngo-pharyngeal carcinoma. The combination of cytotoxic insults at the DNA level (cisplatin), microtubules (docetaxel) and metabolites (5FU) is known to create a synergy that destroys the cancer cells [1-3], which is evident in the initial response leading to regression of the tumour load in 71-80% of the patients [4]. Nevertheless, this high initial response, does not translate into improved loco-regional disease control as almost 40% of the patients are known to develop recurrences [5].

The initial tumour response and subsequent relapse points out

to the presence of heterogeneous groups of cells within the tumour [6]; one of them being the drug resistant subset of Tumour Initiating Cells (TICs) or the cancer stem cells (CSCs) [7-9]. CSCs have been implicated in drug resistance primarily mediated by over-expression of drug efflux protein (*MDR* and *ABC* family genes) [10], dysregulation

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of apoptosis [11] and increased activity of DNA repair gene [12]. An understanding of the association between the CSC behaviour and drug action can provide a better insight into CSC-mediated drug resistance and subsequently the behaviour of patient tumours in terms of treatment response. In head and neck cancer, studies have reported the development of single resistant cell lines [13] which have been used to understand various carcinogenic processes. In this study, we aim to understand the role of CSCs in the process of drug resistance in patient cohorts and *in vitro* models. Studies in patient cohorts were initially carried out to establish the association of the CSCs with drug resistance to combination chemotherapy and prognosis. *In vitro* model systems resistant to a three-drug combination (cisplatin, docetaxel and 5FU) developed previously in the lab [14] were then used to further elucidate the association and to delineate the underlying mechanisms.

Materials and Methods

Patients and tissue samples

Patients diagnosed and treated for squamous cell carcinoma (HNSCC) of oral cavity, larynx, oropharynx and hypopharynx, between December 2009 and January 2012 were accrued retrospectively in this study. Tissue samples were collected from the head and neck bio repository maintained at Mazumdar Shaw Medical Centre (MSMC), Bangalore after obtaining Institutional Review Board (IRB) and Independent Ethical Committee (IEC) approval. Normal samples were collected from patients undergoing dental extraction. All samples were collected after written informed consent. Clinical, demographic and treatment details were obtained from the medical records.

Cell culture

Head and neck squamous cell carcinoma cell lines, CAL-27 and Hep-2 and their corresponding taxol, platinum and 5FU resistant cell lines (CAL-27 TPF and Hep-2 TPF) developed in our lab were used for this study [14]. All the cells were cultured in monolayers with medium containing DMEM supplemented with 10% FBS and 1X penicillin/streptomycin and maintained at 37°C with humidified atmosphere of 5% CO₂. TPF cell lines were maintained in the medium along with the three drugs (cisplatin, docetaxel and 5-FU) at low inhibitory concentrations (IC 6.25).

Drugs, reagents and antibodies

The chemotherapeutic drugs cisplatin (cis-diammineplatinum (II) dichloride), docetaxel, 5FU were purchased from Sigma Aldrich (Sigma Aldrich, St. Louis, MO, USA). The reconstitution of the drugs was according to standard protocols; cisplatin was dissolved in 0.9% sodium chloride while docetaxel and 5FU was reconstituted in DMSO and stored as aliquots at -80°C. Anti-human anti-CD44 monoclonal antibody was purchased from Biogenex (Biogenex, #AM3105M, Fremont, CA, USA) and the secondary antibody anti mouse-Alexa-488 antibody was obtained from Jackson Immuno Research Laboratories (Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA). All the cell culture plasticware, penicillin/streptomycin, recombinant insulin and Tri reagent were purchased from Sigma Aldrich (Sigma Aldrich, St. Louis, MO, USA). High capacity cDNA conversion kit and SYBR green Mastermix were obtained from ABI (Applied Biosystems, Abilene, TX, USA). All cell culture reagents including DMEM, FBS, EGF and b-FGF were purchased from Invitrogen (GIBCO, Invitrogen, Carlsbad, CA, USA).

Stem cell gene expression profiling

Total RNA was extracted from 1×10⁶ cells or from ~ 50 mg of tumor tissues using Tri Reagent and cDNA prepared using the High

Capacity cDNA conversion Kit as per the manufacturer's instructions. Expression profiling of stem cell genes was carried using specific primer sets (Eurofins Scientific, Huntsville, Alabama, USA (Supplementary Table 1) using the Relative Quantification protocol in the ABI Step one system (Applied Biosystems, Abilene, TX, USA). Relative expression profiling was carried out in triplicates and normalized with GAPDH/ β -actin as endogenous controls. The fold level of each marker was calibrated against the expression in the normal oral tissues.

Protein profiling

Immunohistochemical analysis of the patient samples was carried out based on standard protocols using CD44 primary antibody and detected using the Poly-HRP detection system as per manufacturer's instructions (BioGenex, Fremont, CA, USA). Staining was considered positive in the presence of membranous staining (>10%) in the cells. The percentage of positive cells (0-100%) was multiplied with the intensity of staining (mild/1+, moderate/2++, strong/3+++). The CD44 expression score, the maximum being 300 (100% x 3+++). The CD44 expression score in normal mucosa was used as the control.

The expression of CD44 in the cell lines was assessed using immunoblotting; parental and TPF cells were lysed and protein was extracted using MN RNA/Protein Extraction kit (Macherey Nagel, Germany). The proteins were quantified using the Bradford assay and 60µg of each protein was run on a 6% gel and the protein was transferred to nitrocellulose membrane using Transblot apparatus (Biorad, CA, USA). The membrane was blocked overnight and incubated with primary antibody (CD44 and β -actin) for 4 hours. The membrane was washed and incubated with secondary antibody (Goat anti-Mouse) for 1 hour and detected by chemiluminescence.

Flow cytometry analysis

The 70-80% confluent parental and drug resistant cells were trypsinized, re-suspended in the medium with 10% FBS and counted prior to staining for FACS analysis. The required number of cells in phosphate buffered saline (PBS) (1million cells/mL) was incubated sequentially with the primary (anti-CD44) and secondary antibody (anti-mouse Alexa-488, 1:80) at 4°C for 30 minutes each. After each incubation step, the cells were washed with PBS and centrifuged. The cell pellets were then re-suspended in PBS and analysed in a FACS Calibur (BD Biosciences, Franklin, USA). The cells were assessed for fluorescence in the FL1 log channel.

Spheroid formation assay

The sphere forming ability of parental and the resistant cell lines were assessed using protocol described previously [15]. Briefly, both parental and resistant lines were trypsinized and the required number of cells (200 cells/well) re-suspended in serum-free DMEM/F12 medium supplemented with 1% N2 supplement, 20 ng/ml of human recombinant epidermal growth factor (EGF), 5 µg/ml of Insulin (Sigma Aldrich, St. Louis, MO, USA) and of 20 ng/ml human basic fibroblast growth factor (b-FGF) and cultured in 12-well ultra-low attachment plates for two weeks. The medium was supplemented with the growth factors every 3 days. Number of spheroids generated for parental and resistant cells were counted after 12 days. Assays were carried out in triplicates.

Wound healing assay

The migration rate of parental and resistant cells was quantified by the *in vitro* scratch assay as described previously [16]. Briefly, the cells were seeded into 12-well tissue culture plates at a density of 1×10⁵

cells per well. When the cells reached 70-80% confluence, a scratch was made across the monolayer with the 200 µl micro tip. The resulting gap distance therefore equals to the outer diameter of the end of the tip. The wells were replenished with fresh medium and the cells grown for additional 48 hours with periodical recording of wound closure. The wound area was measured using Image J software (NIH, imagej.nih.gov/ij) and the percentage of wound closure evaluated by comparing the wound area at any given time (12, 24, 36 and 48 hours) to the initial wound area (at 0th hour).

Clonogenic survival assay

Colony formation capacities of resistant and parental cell lines were evaluated in the presence and absence of cisplatin using the clonogenic survival assay [17]. Cells were plated in increasing number from 100 to 800 cells per well in a 24-well cell culture plate and allowed to adhere overnight at 37°C. The cells were then treated with a 2-fold increase in concentrations (1 µM to 8 µM) of cisplatin for 72 hours. After removing the drugs the cells were allowed to form colonies for 9-12 days. Colonies were fixed and stained with Giemsa stain (Himedia pvt Ltd, Mumbai, India). Colonies with 50 cells or more were counted manually. The plating efficiency (PE) and the survival fractions (SF) were calculated using the formulae listed below. Survival curves were constructed for determination of survival ability of cisplatin resistant cells relative to parent cells.

PE: Number of colonies/Number of cells seeded.

SF: PE of the treated/PE of the control X100

In vivo tumorigenicity assay

To assess tumorigenicity, the resistant and parental cells were serially diluted into required cell numbers (1×10^5 to 1×10^2) and mixed with 1x Matrigel (BD Biosciences, Franklin Lakes, New Jersey, USA). The cells were then injected subcutaneously (s.c) into the right and left flanks of 5-6 weeks old female/male SCID mice (Severe Combined Immunodeficient, Harlan Laboratories, Indianapolis, USA). A total of three xenografts were generated for each cell number. Tumour formation/growth was assayed weekly up to 5 weeks. The tumour-bearing animals were sacrificed when tumour was large enough ($\leq 1000 \text{ mm}^3$) for subsequent experiments or due to ethical considerations. Animals were taken care of as per the Regulations of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India and Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) compliance. The 'Form B' for carrying out animal experimentation was reviewed and approved by the Institutional Animal Ethics Committee of the collaborating institution (Syngene Pvt. Ltd, Biocon, Bangalore, India).

Histology of xenograft tumours

Xenograft tumours were resected, fixed in formalin and paraffin blocks were made from each of the individual tumour generated from 10^2 and 10^4 cells to confirm the epithelial morphology of the tumour. Tumour sections of 4µm thickness were taken from each tumour block and stained using haematoxylin and eosin. The slides were then reviewed by the pathologist.

Statistical analysis

Statistical analysis was performed by STATA11.1 (College Station, TX, USA). Receiver Operating Curve (ROC) curve analyses was done to evaluate the predictive power of each biomarker with recurrence, the optimal cut point selected based on maximum sensitivity and specificity and the expression of each marker was classified into low and high. ROC curves were then plotted on the basis of the set of optimal

sensitivity and specificity values and the area under the curve (AUC) computed. The biomarker that has the largest AUC was identified as the strongest predictor of recurrence. Pearson *Chi* square test was used to measure the association of each biomarker with recurrence. Logistic regression was carried out to find out the best fit model to predict the outcome using recurrence and non-recurrence as dependant variables. A probability level of $p < 0.05$ was considered as statistically significant.

Comparison between TPF and parental cell lines for sphere formation, wound closure, flow cytometry, CSC marker expression and tumorigenicity was done using Graph Pad PRISM software version 5.00 (graphpad-prism.software.informer.com/5.0, San Diego, CA, USA). Student's *t*-test was used for the statistical analyses of the parental and resistant cell lines and the data were presented as Mean \pm SEM.

Results

Patient characteristics

Two completely independent cohorts of HNSCC patients (N=60) were included as a part of the study; cohort I included patients who were treatment naive (primary tumours) (N=33) and cohort II had recurrent patients who had undergone pre-operative chemo-radiotherapy (CT-RT) before the salvage surgery (N=27) (Table 1). In the salvage group (cohort II), 11 patients received only radiation while 8 each received chemo-radiotherapy (CT-RT), and neo-adjuvant (NAC) chemotherapy. The regimen followed for CT-RT was cisplatin and 5Fu followed by radiation, while NAC included cisplatin, docetaxel and 5FU prior to surgery. All the patients were followed up for a median period of 24

Variables	Outcome	Frequencies
Age (n=60)	Median (Range)	53.5(24-85)
Gender(n=60)	Male	43(71.7)
	Female	17(28.3)
Site (n=60)	Buccal Mucosa	21(35)
	Tongue	16(26.7)
	Other Oral cavity sites	13(21.6)
	Larynx/pharynx	10(16.7)
Habits (n=57)*	Yes	46(80.7)
	No	11(19.3)
T-stage (n=60)	I	7(11.7)
	II	17(28.3)
	III	4 (6.7)
	IV	32(53.3)
N-stage (n=60)	N0	31(51.7)
	N+	29(48.3)
M-stage (n=60)	M0	55 (91.7)
	M+	5(8.3)
Treatment (n=60)	Surgery	33(55)
	Pre Chemo/RT/Surgery	27(45)
Outcome (n=45) Cohort I*	Alive	11(52.4)
	Dead	10 (47.6)
Cohort II*	Alive	13(54.2)
	Dead	11(45.8)
Recurrence/Re-Recurrence (n=48)		
Cohort I*	No recurrence	11(54.2)
	Recurrence	13(45.8)
Cohort II*	No Re-recurrence	15(62.5)
	Re-Recurrence	9(37.5)

Note:* Information unavailable for the remaining patients

Table 1: Summary of Clinical Characteristics of Patients.

months (range 6 to 44 months). In the primary cohort, 9 patients were lost during follow up.

Expression profiling of CSC markers in HNSCC

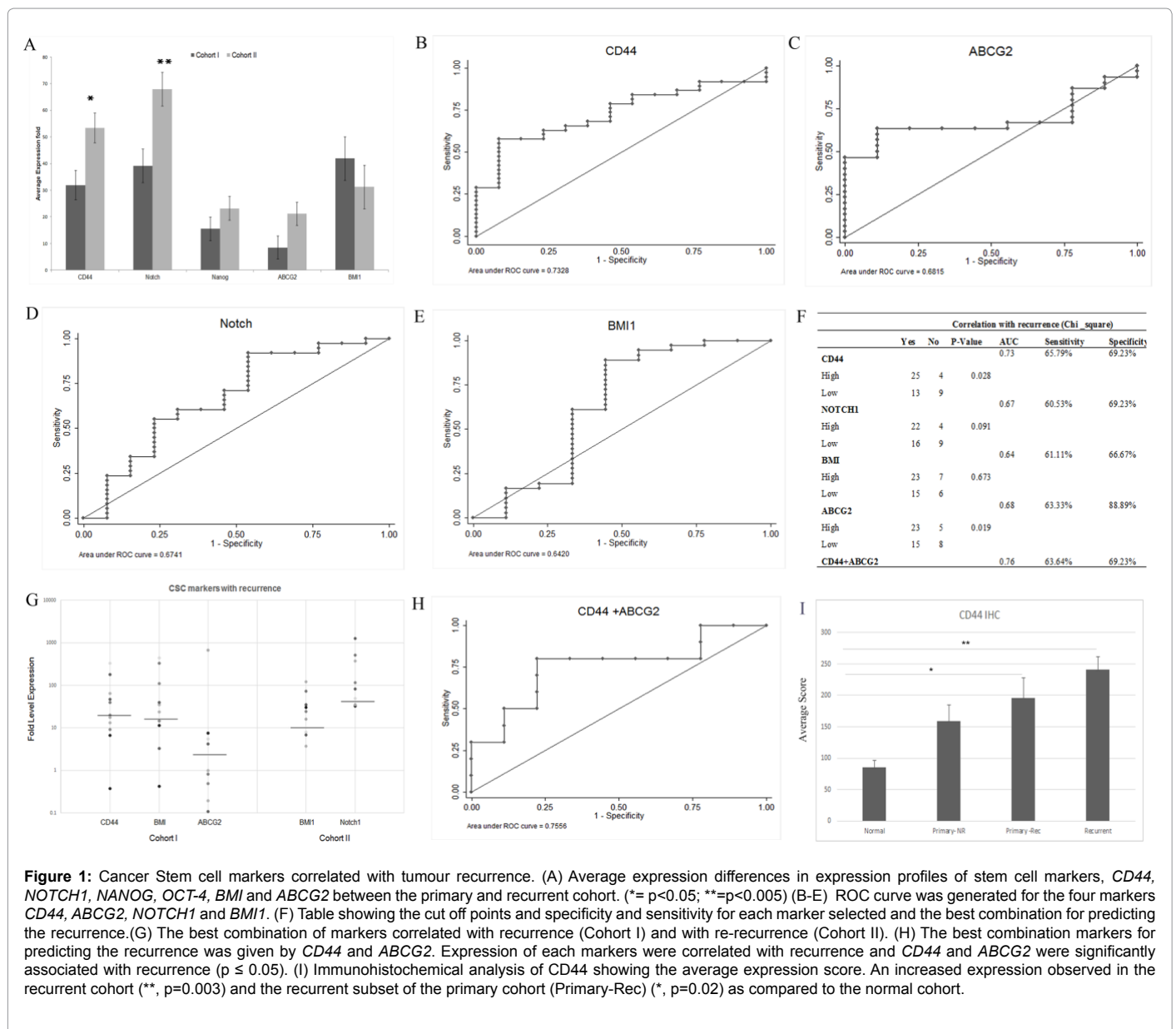
Gene expression profiling for stem cell markers such as *CD44*, *CD133* (Prominin1), *OCT-4* (*POU5F1*), *NANOG*, *ABCG2* (*BCRP1*), *BMI1* (B cell-specific Moloney murine leukaemia virus integration site 1) and *NOTCH1* was carried out in the two patient cohorts and compared with the normal controls (N=3). A majority of the genes analysed (5/7) showed an average increase in their expression in recurrent cohort as compared to the treatment naïve subset; *ABCG2* (2.5 fold; 21.2 ± 7.3 vs. 8.4 ± 4.3), *CD44* (1.7 fold; 53.4 ± 7.8 vs. 31.9 ± 5.5 ; $p=0.024$) and *NOTCH1* (1.7 fold; 67.9 ± 6.8 vs. 39.1 ± 6.3 ; $p=0.002$) showed the highest fold difference. *NANOG* (1.5 fold; 23.1 ± 6.6 vs. 15.5 ± 4.4) showed a comparatively lower fold increase while *BMI* expression was higher in the treatment naïve cohort (1.3 fold; 41.9 ± 8.1 vs. 31.2 ± 1.2) (Figure 1A).

OCT-4 and *CD133* genes remained undetected in the normal cohort and hence relative quantification of expression was not carried out. A comparison between the two patient cohorts indicated that *CD133* was detected in a higher percentage of recurrent patients (52% vs. 42%) as compared to cohort 1.

CSC marker expression and disease prognosis

Based on the expression fold analysis, four markers *CD44*, *NOTCH1*, *BMI* and *ABCG2* were further assessed using ROC analysis (Figure 1B-1E). The patients were classified as low/high for each marker based on the ROC and the association of CSC marker expression with recurrence was then evaluated (Pearson *chi*-square test). *CD44* ($p=0.028$) and *ABCG2* ($p=0.019$) correlated with recurrence in the entire cohort. *Notch1* was showing a trend of near significant association with recurrence ($p=0.091$) (Figure 1F).

Expression of these CSC markers was also significantly associated



with the incidence of disease recurrence/re-recurrence in the two cohorts. In cohort I, the four genes showed a high correlation with the recurrence status with more than 30% of the patients having a high expression of these genes (*BMI*^h: 60% (6/10), *CD44*^h: 54.5% (6/11), *ABCG2*^h: 44% (4/9) and *NOTCH1*^h: 36.4% (4/11)) (Supplementary Table 2). Among these patients, 63.6% (7/11) were positive for at least two markers and the best marker combinations being *CD44*, *ABCG2* and *BMI* (Figure 1G).

In cohort II, the re-recurrent cases had a high expression of one of these markers (*ABCG2*^h: 71.4% (5/7), *BMI*^h: 66.7% (6/9), *NOTCH1*^h: 66.7% (6/9), *CD44*^h: 44.4% (4/9)) (Supplementary Table 2). 88.9% (8/9) of the re-recurrent patients had an increased expression of at least two markers, the best combination being *BMI* and *NOTCH1* (44.4% cases high for both markers) (Figure 1G).

All these markers when analysed by logistic regression (Breslow Method) gave positive coefficient values with recurrence, *CD44* having the highest hazard ratio (HR=3.18; 95% CI 0.81–12.51) followed by *ABCG2* (HR=1.70; 95%CI 0.40– 7.26), *BMI* (HR=1.91; 95% CI 0.40 – 9.24) and *NOTCH1* (HR=1.59, 95%CI: 0.45–5.63). In an effort to find the best combination of marker for predicting recurrence in the

treatment naïve cohort, a multivariate classification model was built using these four markers and a combination of *CD44* with *ABCG2* gave the best ROC (AUC: 0.76) (Figure 1H).

Immunohistochemical analysis of *CD44* in the patient cohorts indicated a significant difference in the average score of the cohort II (N=19; 240.59 ± 20.86) as compared to the non-recurrent subset of cohort I (N=11; 159±25.56; p=0.02) and the normal controls (N=4; 85±10.9; p=0.0031). In addition, the subset of patients in cohort I which recurred later also had a significant up regulation of *CD44* (N=9; 196.26±32.01; p=0.04) indicating the prognostic significance of the marker (Figure 1I and Figure S1A-D). In an effort to further understand the association between the CSC behaviour and drug resistance/recurrence, the triple drug resistant model systems were further characterized.

TPFR cell lines were enriched with CD44 positive cells

The triple drug resistant cell lines (Hep-2 TPFR, CAL-27 TPFR) generated as described previously [1] were assessed for the CD44+ cell population by FACS analysis. Both the resistant cell lines showed a marginal increase in the CD44+ cells. While CAL-27 TPFR (86.8

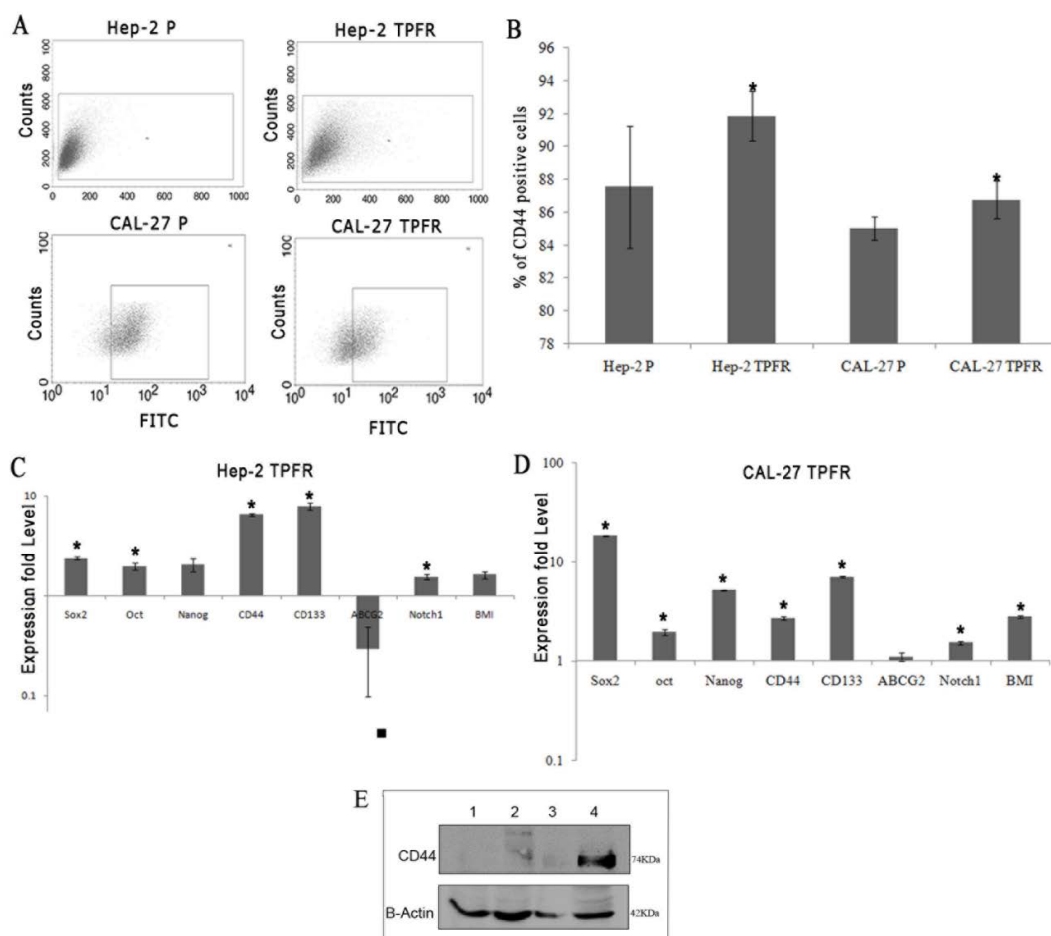


Figure 2: Resistant cell lines show up regulation of stem cell expression (A-B). The percentage of CD44 positive cells were detected in parental and TPFR cell lines using FACS and the results are represented from 3 independent experiments. Both Hep-2 and CAL-27 TPFR cells showed a marginal increase in CD44 subpopulation relative to the parental cells. (C&D): The gene expression profile of a panel of stem cell markers in Hep-2 and CAL-27 TPFR cells. In Hep-2 TPFR cells the median increase of stem cell genes was 2.01 fold (0.28 to 7.86) and that in CAL-27 TPFR was 2.73 (range: 1.1 to 2.73). The statistically significant differences in expression are indicated (*). (E) Immunoblotting in CAL-27 P and Hep-2 P (Lanes 1 & 3) and TPFR cells (Lanes 2 & 4) showed highest level of expression in the Hep-2 TPFR cell lines. B-actin expression is shown as the control.

± 1.16) cells showed a 1.8% over the parental cells (85 ± 0.70), Hep-2 TPFR (91.87 ± 1.52) showed a 4.3% increase in the percentage of CD44+ cells compared to the parental CAL-27 (87.6 ± 3.7) respectively (Figure 2A and 2B).

Enhanced expression of CSC markers in Hep-2 TPFR and CAL-27 TPFR

Resistant cell lines were profiled for the expression of CSC markers such as *CD44*, *ABCG2*, *NOTCH1*, *BMI1*, *CD133*, *SOX2*, *OCT-4* and *NANOG* at the transcript level. Quantitative profiling of the transcript levels indicated a median 2-fold increase in the expression of all markers except *ABCG2* in the resistant cells lines versus parental cells (Hep-2 TPFR: 2.09 fold; range: 0.28 to 7.86; CAL-2 TPFR: 2.73 fold; range: 1.1 to 18.3). *CD133*, *CD44*, *OCT-4*, *SOX2*, *BMI*, *NANOG* and *NOTCH1* were significantly ($p < 0.05$) up regulated in Hep-2 TPFR cells; *CD44* showed a highly significant elevation of expression ($p < 0.0001$). In CAL-27 TPFR, the up regulation of *SOX2*, *NANOG* and *CD133* in CAL-27 TPFR was more significant ($P < 0.0001$) as compared to

OCT-4, *NOTCH1*, *CD44* and *BMI* ($p < 0.05$) (Figures 2C and 2D). Immunoblotting of the resistant cell lines with CD44 also showed an increased expression of the gene in Hep-2 TPFR cells as compared to the parental and CAL-27 TPFR (Figure 2E).

TPFR cells were more migratory and proliferative

The migratory potential of TPFR cell lines was confirmed by the wound healing assay. The percentage of wound closure was calculated for different time periods for each cell line and compared with that of the parental. Quantification of the wound closure between the two resistant cell lines showed that the Hep-2 cells showed a slower overall migration rate. The Hep-2 TPFR cells closed the wound by 50% and 83% after 24h and 30h respectively, the difference being highly significant at both the time points (24h: $p = 0.0038$; 30h: $p = 0.002$) when compared to the parental cells (Hep-2 P) (11% and 42%). Hep-2 TPFR completely closed the wound by 48h but Hep-2 P closed only 64% of the wound at the same time point (Figures 3A-3C).

The increase in the migration potential was more significant in the

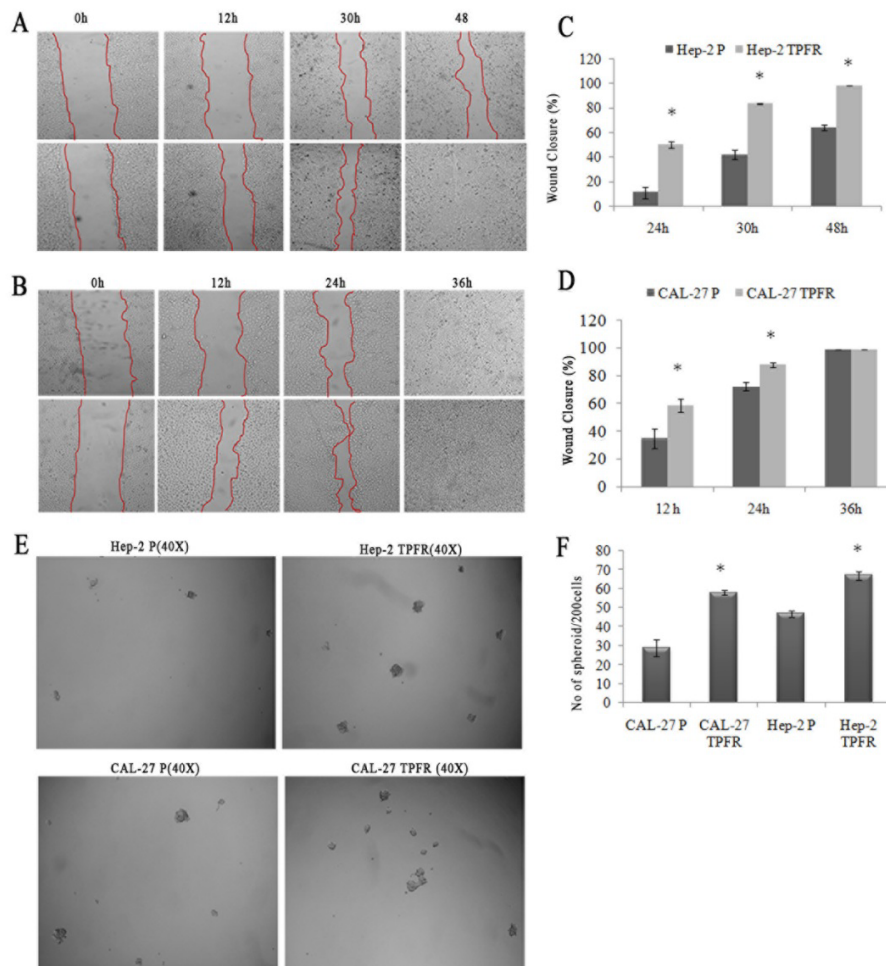


Figure 3: TPFR cells showed increased migratory and self renewal properties (A&B). The migratory ability of Hep2-TPFR and CAL-27 TPFR cells was assessed by the wound healing assay. (A) The closure of Hep-2 parental cells (Upper panel) and TPFR (Lower panel) was compared at different time points of 0, 24, 30 and 48 h. At 48 h, Hep-2 TPFR completely closed the wound as compared to 64.14% of closure by its parental cells. (B) The closure by CAL-27, and the TPFR was documented from 0-36 h. CAL-27 TPFR and parental cells closed the wound by 36 h but the percentage wound closure by CAL-TPFR at 12h and 24 hr was higher than that of its parental cells (Magnification is 200X). Graphical representation of the percentage of wound closure (SEM \pm SE, three independent experiments, $p \leq 0.05$) at different time points is also shown (C & D). The number of spheroids (40X) generated by Hep-2 TPFR cells (upper panel) and CAL-27 TPFR (Lower panel) in low attachment serum free condition was more than their parental cells (E). Graphical representation of the results are shown (F) as SEM \pm SE of three independent experiments.

Hep-2 TPFR cells when compared to CAL-27 TPFR. CAL-27 TPFR cells showed a significant difference in the wound closure at different time points. At the end of 12 hours, 58% ($p=0.0275$) of the wound was closed as compared to 34% by the parental cells. The difference in migratory potential was maintained after 24 hours wherein 88% ($p=0.0498$) of the area was closed by the TPFR cells while CAL-27P (parental cells) closed the area by 72%. At 36h both parental and resistant cells closed the wound area completely (Figures 3B and 3D).

TPFR cells show increased spheroid formation and self-renewal property

The spheroid formation ability of the TPFR resistant cells was compared with that of parental cells. Hep-2 TPFR cells (67.5 ± 2.2) showed a significant (1.4 fold, $p=0.002$) increase in the spheroid formation ability as compared to the parental (47.3 ± 1.78). CAL-27 TPFR (58.3 ± 4.3), on the other hand showed a 2-fold increase in the number of spheroids (CAL-27 P: 29.7 ± 1.2) ($p=0.003$) (Figures 3E and 3F).

TPFR cells were more clonogenic on drug exposure

The survival ability of the resistant cell lines after cisplatin treatment was assessed by clonogenic survival assay. In both the resistant cell lines, there was significantly higher number of surviving colonies from 1 μM to 8 μM concentrations. CAL-27 TPFR cells showed significantly higher surviving fractions at 2 μM (77.2 ± 2.8 vs. 12.8 ± 1.2 ; $p<0.0023$) and 4 μM (33.3 ± 3.3 vs. 4.6 ± 0.94 ; $p=0.001$) of cisplatin. This difference was not significant at 8 μM of the drug (5.6 ± 0.72 vs. 3.5 ± 0.5 , $p<0.069$). However, Hep-2 TPFR showed a significantly higher number of surviving clones (66.3 ± 2.9 ; $p<0.01$; 31.4 ± 0.98 ; $p<0.01$ and 10.7 ± 0.57 ; $p<0.03$) at 1.25 μM , 2.5 μM and 5 μM of cisplatin as compared to the parental cells (10.9 ± 1.6 , 6 ± 1.5 and 5.9 ± 1.36) (Figures 4A-4D).

The drug resistant cells were more tumorigenic than the parental cells

The tumorigenic potential of TPFR cell lines was confirmed in an *in vivo* tumour xenograft model. Hep-2 parental and TPFR with different cell number ranging from 10^5 to 10^2 ($N=3$ for each cell number) were injected subcutaneously (s.c) into the right and left flanks of each animal. The tumour volume was recorded from the day a palpable tumour was observed (Day 14) and all tumours reached a volume of >300 mm³ within 5 weeks of time. Hep-2 TPFR cells showed an increased tumour burden at end of the study as compared to the parental cells, though the difference was not statistically significant (Figure 4E). At 10^2 cells, Hep-2 P was able to form tumour in lesser number of animals (1/3) as compared to TPFR (2/3). In addition, the increase in tumor burden at the end of 44 days was higher in the TPFR arm (9.6 fold vs. 6 fold) indicating increased tumorigenicity at this cell number (Figure 4F). The H&E of the tumour sections made from 10^4 and 10^2 cells of each cell line confirmed the squamous cell morphology of the tumours (Figures 4G-H).

Discussion

Understanding resistance to chemotherapy is one of the major steps to improve the outcome of head and neck cancer patients. Cancer Stem Cells (CSCs), as is the case with their normal counterparts, are drug resistant in nature and hence the concept that these cells might be responsible for disease relapse/recurrence has gained significance. Studies in different solid cancers have reported a possible association of stem cell markers with prognosis [18]. Parallel investigations have also recently shown that stem cell markers are associated with resistance [19,20] and that continuous/short term exposure to chemotherapeutic drugs in culture can lead to the enrichment of stem cell-like cells in

lung, prostate and ovarian cancers [21-23]. In this study we establish the association of CSC specific markers with disease recurrence and their prognostic efficacy in head and neck cancer patients treated with combination chemotherapy. We also demonstrate the effect of combination chemotherapy on cancer stem cell behaviour in patients and triple drug resistant HNSCC cell line models.

In our study, we observed a significant correlation of the CSC markers, *CD44* and *ABCG2* with disease recurrence and these markers in combination, predicted poor prognosis in the patients. Accumulating evidences do provide similar evidence in breast cancer; markers of tumour initiating cells could predict the response to chemo-radiotherapy [24,25]. Studies in laryngeal cancer have also reported that CSCs characterised by multiple markers such as *CD44*, *CD24* and *OCT4* have correlated with local recurrence post radiotherapy and chemo-radiotherapy [26,27]. Studies from our lab have also shown the association of *CD44+* cells at the invasive tumor front to be associated with prognosis of patients (unpublished data). The association of a stem cell gene signature with drug resistance and poor prognosis has also been reported in cancers of other sites such as breast and prostate cancers [24,28]. These evidences along with the results of this study indicate that expression levels of CSC-specific markers correlate with disease recurrence and overall prognosis. In addition, our study also indicates that *CD44*, *ABCG2* and *BMI1*, in combination are arguably the best markers associated with recurrence in a treatment naïve primary cohort while *BMI1* and *NOCTH1* are poor prognosticators in a recurrent setting; further prospective evaluation with a higher sample size will establish the clinical utility of these markers.

In vitro culture systems are excellent models to delineate the possible mechanisms underlying many carcinogenic processes, one of them being drug resistance. Studies carried out with cancer cell lines have recently shown that continuous/short term exposure to single treatment leads to the enrichment of stem cell-like cells in culture [22,23]. In our study, an increase in the expression of the CSC markers in the recurrent cohort of patients exposed to combination chemotherapy and/or radiation indicated their enrichment post treatment. This was further reflected in the expression profile of the cell lines resistant to a combination of cisplatin, docetaxel and 5FU [14]. The enrichment of CSCs was further substantiated at a functional level with an increase in their self-renewal, migratory and tumorigenic capacity. These observations indicate that exposure to the TPF combination of drugs leads to enrichment of the resident cache of CSCs. An understanding of the markers that signify this enrichment and the associated mechanisms leading to drug resistance will be a step towards applying this knowledge for clinical benefit.

Multi-drug resistance in normal as well as cancer stem cells is known to be accomplished by a wide range of mechanisms ranging from increase in drug efflux, improving survival to having more efficient repair mechanisms. The induction of the stem cell related pathways in itself is known to activate these drug resistant mechanisms. As mentioned previously, our study showed that *CD44* and *ABCG2* correlated with recurrence in the patient cohort with *CD44* showing a similar trend of over expression post drug treatment in the resistant cell lines. The functional network between the *CD44*, its ligand Hyaluronic Acid (HA) and the subsequent activation of the ABC group of drug efflux proteins is probably the mainstay behind CSC-mediated drug resistance [29-32]. The HA-*CD44* interaction is also known to activate anti-apoptotic pathways like *PI3K/AKT* and *MAPK/ERK*, subsequently leading to induction of the ABC family proteins [33,34] as well as survival pathways (Survivin). This finding is further supported by studies in breast tumours [35]. *BMI1*, the other gene that was significant in our study was not enriched post therapy in patients,

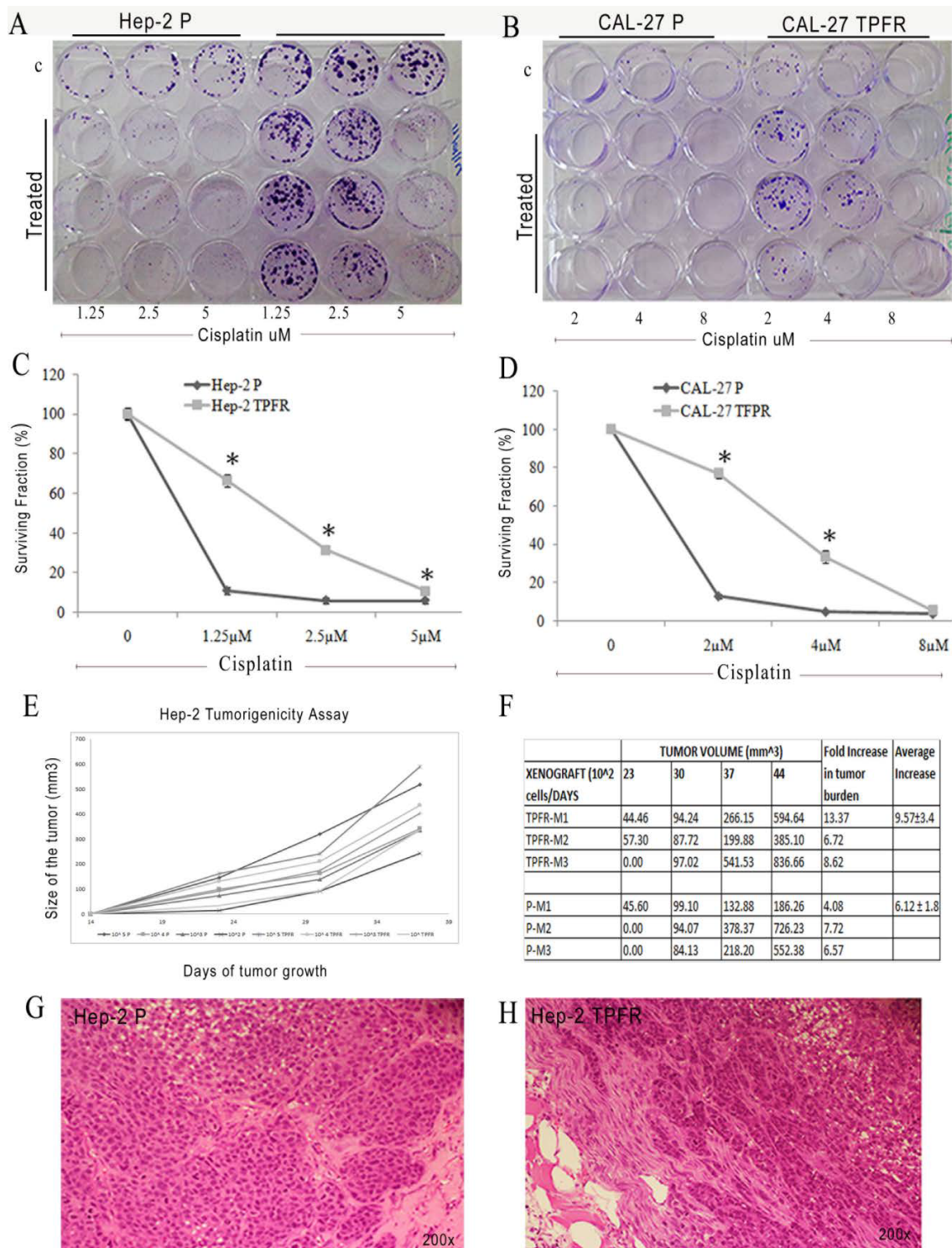


Figure 4: TPFR cells showed increased clonogenic and *in vivo* tumorigenic properties (A&B) The number of clones survived after cisplatin treatment in Hep-2 (A) and Cal-27 (B) P and TPFR cells. Hep-2 TPFR (Lane 4-6: 1.2uM, 2.5 uM & 5 uM) cells were more clonogenic than Hep-2 P cells (lane: 1-3 treated). CAL-27 P (Lane 1-3) and TPFR cells (lane 4-6) when treated with increasing concentration of cisplatin (2uM, 4uM & 8uM), TPFR cells produced more number of clones. In both the cell lines increasing cell numbers were used in each lane; 200 (lane 1, 4), 400 (lane 2, 5) and 800 (lane 3, 6). The controls (c) lane represented untreated cells of CAL-27 P and TPFR cells (100 cells). (C&D): Survival fraction of colonies are presented as graphs for Hep-2 and CAL-27 parental and TPFR cells. Statistically significant results are indicated by asterisk (p<0.05) (E) Tumour burden of the Hep-2 TPFR cells: serial cell numbers from 10⁵ to 10² cells mixed with matrigel were injected subcutaneously into the SCID mice and tumour formation was observed for 37 days. TPFR cells showed increased tumour burden with all cell numbers injected. (F) The table representing the tumour volume/ at 10⁴ cells at different time points (N=3) till 44 days. The representative H&E staining (magnification 200X) of the tumour is showing the histology of the tumours generated by Hep-2 P (G) and Hep-2 TPFR (H).

but correlated to recurrence/re-recurrence in both the cohorts; studies in different cancers have shown a BMI-driven signature to be associated with poor survival and lack of response to chemotherapy [36,37]. BMI is also known to exert its effect on the PI3/AKT pathway and thereby induce other drug resistance pathways [38]. Contrary to the results observed in patients, this gene was up regulated in TPF cell lines suggesting a possible role for this marker in acquired drug resistance. Our previous study has shown an up regulation of *MDR-1*, *MRP2* as well as *Survivin* in both these TPF cell lines [14]. This, in combination with the enrichment of stem cell markers reported in this study, indicates that acquired resistance to combination chemotherapy is probably achieved by induction of these molecules through multiple CSC-mediated pathways.

The expression of *NOTCH1* and *CD133* was also high in recurrent patients with a significant up regulation in the TPF cells. High levels of *CD133* expression along with other cancer stem cell markers has been found to be an independent predictor of tumour recurrence after chemotherapy in HNSCC [39] and other cancers [40,41]. Studies in HNSCC tumours and cell lines documented the association of NOTCH1 and CD133 activity with Cisplatin resistance [42,43]. Activation of NOTCH1 is reported to impart chemo resistance in prostate CSCs by activation of ABCG1 [44] while CD133 is known to influence resistance through ABCB1/MDR1 [10]. The combined effect

of all these pathways on the drug transporters is probably reflected in the extremely high up regulation of *MDR1* and *MRP2* observed in the TPF cell lines [14]. This indicates that despite the synergistic, cytotoxic response initially obtained by the use of the TPF combination of drugs, a parallel enrichment of CSCs may lead to the acquisition of resistance through multiple pathways, which in turn might result in a relapse of the tumour.

In addition to the enhanced self-renewal and migratory properties, the TPF cells display an increased colony forming efficiency despite a high concentration drug assault. This suggests that in patients resistant to these drugs, a continued exposure can lead to the generation of a more aggressive subset of tumour initiating cells, ultimately leading to extensive disease relapse/recurrence. This correlation was further evident in the recurrent patient cohort of this study, wherein more than 80% patients with multiple episodes of tumour recurrence had an up regulation of two or more CSC markers. A recent report showing that increased levels of ALDH1, an indicator of stem cells, post radio chemotherapy in rectal cancers predicts poor prognosis [45] further emphasizes this concept. Studies in non-small cell lung cancer (NSCLC) have also indicated that the tumour re-initiating cells (TRICs) surviving chemotherapy, have a higher EMT-like behaviour as compared to a general CSC characteristic, and can lead to more invasive/aggressive tumours [46]. Contrarily, the cells resistant to drugs

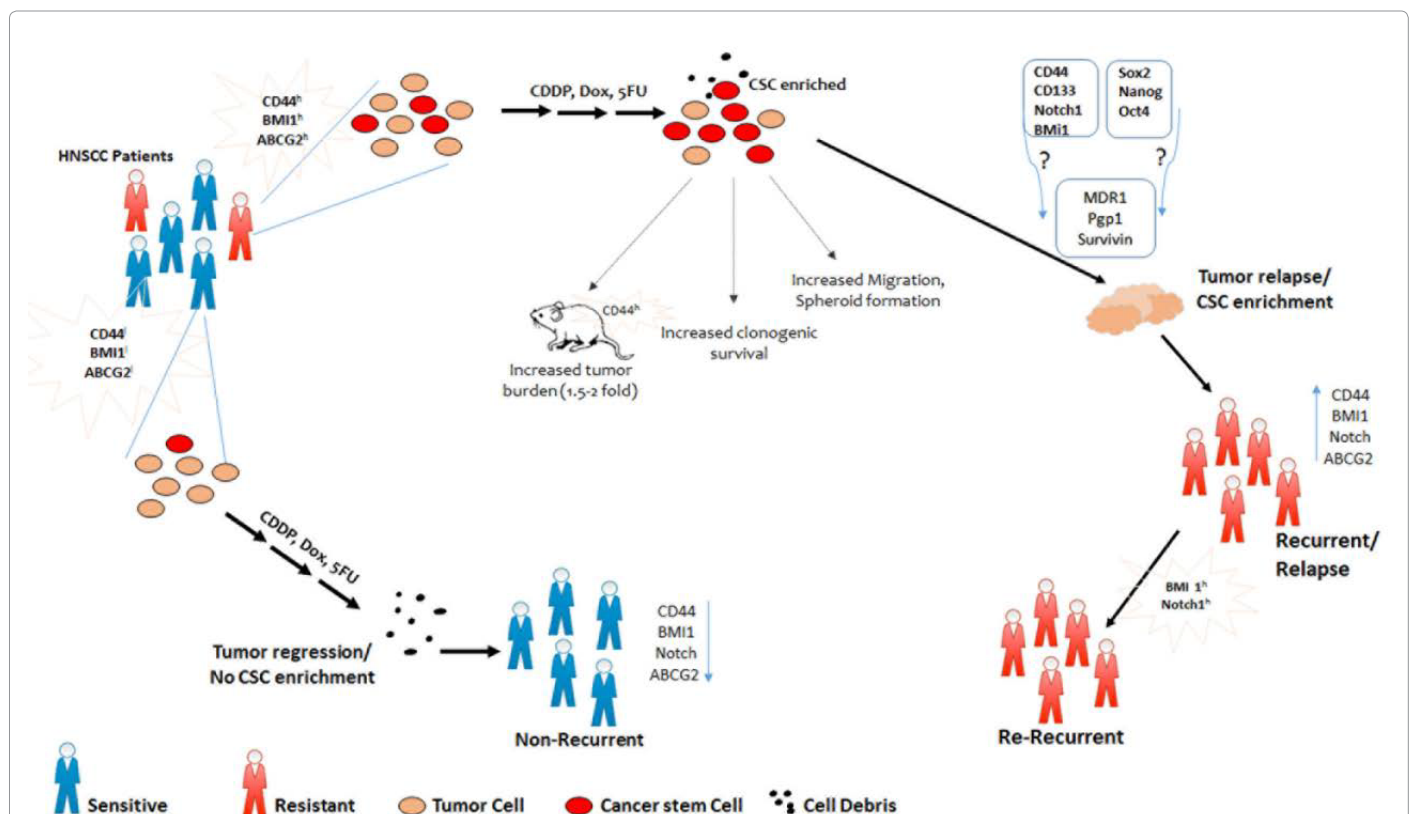


Figure 5: Cancer stem cells, Drug Resistance and Prognosis in HNSCC. The possible role of CSCs in the process of drug resistance is explained in the model. Increased expression of specific markers in the treatment naive patients can indicate poor outcome; recurrence ($CD44^+/ABCG2^+/BMI1^+$) or no recurrence ($CD44^+BMI1^-/ABCG2^-$). Combination chemotherapy on patients resistant to the drugs leads to an enrichment of the resident cache of CSCs in the resistant patients. These cells with an enrichment of CSCs have an increased self-renewal, migration and clonogenic survival with capacity to generate a higher tumour burden in SCID mice. These cells also showed an increase in CSC markers (CD44, CD133, Notch1 and Sox2-Oct4) and survival pathways (MDR1/Pgp1/Survivin) suggesting that the possibility of a CSC-mediated drug resistance. These drug resistant CSCs ultimately lead to generation of more aggressive tumours and lead to a disease relapse in the resistant patients. In contrast, in the ($CD44^+BMI1^-/ABCG2^-$) cohort, combination chemotherapy does not lead to enrichment of the CSCs and is thereby effective in controlling the disease.

in our study showed a high CSC-related behaviour, but the tumorigenic capacity (though an increase in tumour burden was observed) was not very different as compared to the parental cells. These cells do exhibit a higher migratory capacity; whether these cells do have other EMT characteristics and can lead to more aggressive/invasive tumours needs to be explored further, a line of thought currently being pursued in the lab.

In conclusion, data from our patient cohort and the resistant cell line models demonstrate a clear association between the enrichment of CSC-like cells post combination chemotherapy. The association of CSCs with drug resistance/disease recurrence and subsequently prognosis in head and neck cancers can be explained by a possible model. In this study, in both the treatment naïve and recurrent cohorts, increased CSCs, as indicated by *CD44/BMI1/ABCG2*, signified poor prognosis (Figure 5). This indicated that a certain threshold level of CSCs might be a determinant of prognosis. Treatment of the HNSCC patients by combination therapy leads to an enrichment of this resident CSCs in the resistant (*CD44^h/BMI1^h/ABCG2^h*) patients. The enhanced self-renewal, migratory and clonogenic survival of drug resistant CSCs points out that these cells can lead to the generation of increasingly aggressive tumours in these patients, leading to a disease relapse. On the other hand, in the CSC^{low/-}, drug sensitive cohort, treatment regresses the tumour and due to an absence of a parallel CSC enrichment, does not lead to tumour relapse. Molecular profiling also suggested that continuous exposure of cytotoxic drugs in resistant patients, can lead to activation of CSC-mediated drug resistance pathways; activation of survival and drug transporter pathways (Survivin^h/PgP^h/MDR^h) and a possible development of invasive tumours through an EMT mediated phenotype. Studies are currently ongoing in the lab to delineate these suggested mechanisms of drug resistance and to further understand the differences observed due to single and combination therapy. These studies will be a step towards identification of CSC-driven therapeutic approaches that can be adopted in patients resistant to conventional cytotoxic therapies.

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