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Proportion of CD44$^+$ Subset of Tumour Cells in Single Cell Suspension Prepared from FFPET Sections Directly Correlates with Histological Subtyping of Head and Neck Squamous Cell Carcinoma

(Bahagian CD44$^+$ Subset Sel Tumor dalam Suspensi Sel Tunggal Disediakan daripada Bahagian FFPET Berkorelasi Terus dengan Pengetipan Histologi Karsinoma Sel Skuamosa Kepala dan Leher)

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ABSTRACT

CD44 expression in tumours imparts potential to progress, metastasize, recurrence, and resistance against antineoplastic therapy. In this study, we sought to describe the variation in the immuno-expression and numeration of MDR1$^+$ and CD44$^+$ potential cancer stem cells in different histological grades and subtypes of head and neck squamous cell carcinoma (HNSCC). Flow-cytometric analysis was performed on single cell suspension prepared from formalin fixed paraffin embedded tissue (FFPET) sections of HNSCC using anti-CD44 and anti-MDR1/ABCB-1 primary monoclonal antibodies. Immunohistochemical (IHC) staining was also carried out using both of these antibodies on HNSCC tissue sections mounted on super frosted glass slides. On immunohistochemical analysis, the mean IRS for CD44 and MDR1 were 8.6364 ±3.02114 and 1.5909 ±1.27674 respectively. When mean immune-expression scores of CD44 antibody and MDR1/ABCB-1 were compared with histological grades and subtypes of HNSCC, the relationship was found to be statistically insignificant. Interestingly, a strong statistical difference (p = 0.000) was observed when the mean score of subset of dysplastic squamous epithelial cells with characteristics of cell stemness (CD326$^+$CD44$^+$) was compared among different histological subtypes of HNSCC using flowcytometric analysis. While no statistically significant association was observed when the mean score for subset of dysplastic cells with potential of drug resistance (CD44$^+$MDR1$^+$) was compared among different histological subtypes of HNSCC. Although potential cancer stem cell marker CD44 and the multidrug resistance maker MDR1/ABCB co-expressed in HNSCC but the proportion of CD326$^+$CD44$^+$ subset of tumour cells (potential cancer stem cells/CSCs) significantly correlates with least aggressive to more aggressive tumour subtypes.

Keywords: CD44; CSCs; FFPET; flowcytometry; HNSCC; immunohistochemistry; multidrug resistance; OSCC

ABSTRACT

Pengekspresan CD44 dalam tumor memberikannya potensi untuk berkembang, bermetastasis, berulang dan berintangan terhadap terapi antineoplastik. Dalam kajian ini, kami ingin menerangkan variasi dalam pengekspresan imun dan penbilangan sel stem kanser berpotensi MDR1$^+$ dan CD44$^+$ dalam gred histologi yang berbeza dan pengetipan karsinoma sel skuamosa kepala dan leher (HNSCC). Analisis aliran-sitometri dilakukan pada penggantungan sel tunggal yang disediakan daripada bahagian tisu terbenam parafin tetap formalin (FFPET) HNSCC menggunakan antibodi monoklonal utama anti-CD44 dan anti-MDR1/ABCB-1. Pewarnaan imunohistokimia (IHC) juga dilakukan menggunakan kedua-dua antibodi ini pada bahagian tisu HNSCC yang dipasang pada slaid kaca super beku. Pada

analisis imunohistokimia, purata IRS untuk CD44 dan MDR1 masing-masing ialah 8.6364 ±3.02114 dan 1.5909 ±1.27674. Apabila min skor pengekspresan imun antibodi CD44 dan MDR1/ABCB-1 dibandingkan dengan gred histologi dan pengetipan HNSCC, hubungan itu didapati tidak signifikan secara statistik. Menariknya, perbezaan statistik yang kuat ($p = 0.000$) diperhatikan apabila skor min subset sel epitelium skuamosa displastik dengan ciri-ciri pensteman sel ($CD326^+CD44^+$) dibandingkan antara pengetipan histologi HNSCC yang berbeza menggunakan analisis sitometri aliran. Walaupun tiada perkaitan yang signifikan secara statistik, diperhatikan apabila skor min bagi subset sel displastik dengan potensi rintangan drug ($CD44^+MDR1^+$) dibandingkan antara pengetipan histologi HNSCC yang berbeza. Walaupun penanda sel stem kanser berpotensi CD44 dan pembuat rintangan pelbagai drug ABCB-1/MDR1 dinyatakan bersama dalam HNSCC tetapi perkadaran subset $CD326^+CD44^+$ sel tumor (sel stem berpotensi kanser/CSC) berkorelasi dengan ketara dengan tumor yang paling tidak agresif kepada pengetipan tumor yang lebih agresif.

Kata kunci: CD44; CSCs; FFPET; HNSCC; imunohistokimia; OSCC; rintangan pelbagai ubat; sitometri aliran

Introduction

HNSCC constitutes 90% of the malignancies of the head and neck region. The reported incidence of this cancer categorizes it 9th among all cancers worldwide (Gupta, Johnson & Kumar 2016). HNSCC has diverse biological patterns and is also very heterogeneous genomically. It arises from stratified squamous epithelial linings of the upper aerodigestive tract and is categorized based on the anatomical locations of HNSCC. Squamous cell carcinomas of facial skin, lip, oral cavity, pharynx, larynx, hypo-pharynx, nasal, and paranasal sinuses are collectively called HNSCC (Adelstein et al. 2017). The histopathological spectrum of HNSCC is determined by the extent of squamous differentiation and cellular atypia, as it arises from the stratified epithelium of the upper aerodigestive mucosa. When a tumor is well-differentiated, its cells have a mature appearance and are arranged in layers with irregular keratinization, resembling the stratified epithelium. These tumors often display a 'keratin pearl' appearance. On the other hand, a tumor that is poorly differentiated is distinguished by the presence of immature cells with nuclear pleomorphism and atypical mitoses. These tumors show little to no organized stratification or keratinization (Johnson et al. 2020). HNSCC can be categorized into different histological subtypes, including conventional squamous cell carcinoma (CSCC), verrucous carcinoma (VC), and basaloid squamous cell carcinoma (BSCC), with CSCC being the most common subtype (Scully & Bagan 2009).

This disease is considered to be one of the highly metastatic cancers with low survival rates because patients with HNSCC present with locally advanced disease in 50% of the cases with very poor disease-free survival time (Bhave, Teknos & Pan 2017). To optimize the treatment outcome and enhance the survival time for these patients, treatment selection should be done carefully and biomarkers should be discovered for the monitoring of treatment response and early detection if recurrence happens to improve the patient's outcome (Arantes et al. 2017). Surgery alone or a combination surgery with adjuvant chemo-radiotherapy, are currently the treatments of choice for HNSCC patients. However, very marginal improvement has been reported during the last few decades in terms of disease-free 5-year survival rate which is still below 50% for HNSCC patients (Chen & Wang 2019).

CD44 belongs to a family of type I trans-membrane glycoproteins which are involved in cell-cell interaction, adhesion of cell, and also a key factor in cell migration (Mărgăritescu et al. 2011). The binding of this CD44 with a non-sulfated and anionic glycosaminoglycan called hyaluronic acid (HA) is a crucial step in driving the biological activities of this trans-membrane glycoprotein. These biological activities set their role in promoting carcinogenesis by managing adhesion, migration, proliferation, and invasion of dysplastic cells (Ghosh, Alpay & Klostergaard 2012). The expression of CD44 parallels with increased angiogenesis, poor histological grade of tumour, lymphatic involvement, metastasis, treatment resistance and deteriorated prognosis in HNSCC patients (Garcia et al. 2019). CSCs were initially defined based on the membranous expression of $CD44^{bright}$ and $CD44^{dim}$ populations on flowcytometric analysis. Prince et al. (2007) reported that cells with CD44 bright expression (5×10^3) can regenerate tumor heterogeneity and are capable of forming a new tumour in immune-compromised mice as compared to $CD44^{dim}$ cells (5×10^5) which lack these hallmarks of malignant potential. Given the above-mentioned findings, several researchers have claimed that subpopulations of cells positive for CD44, either in cancer cell lines or primary cell culture of HNSCC, show an enhanced proliferation and migration

potential, or also demonstrate an increased capacity to sphere formation, invading normal tissues and to resist against chemotherapy drugs (Joshua et al. 2012; Sterz et al. 2010; Su et al. 2011).

Multi-drug resistance (MDR) is an important feature of HNSCC in which tumour cells resist chemotherapy (Yang et al. 2015). MDR can be explained based on its dependency on multiple biological factors but drug efflux proteins play a major role and are frequently linked with it. The normal body cells have a well-regulated drug efflux mechanism and these efflux transporter proteins are embedded in plasma membranes. There is solid evidence that the same transporter proteins may also be involved in the efflux of chemotherapeutic drugs from CSCs to the outside of CSCs. An important family of transporter proteins, known as the ATP-Binding Cassette (ABC), comprises several transporters, which when expressed in high numbers on the plasma membrane, may drive anticancer drugs out of cancer cells into the extracellular environment and play a vital role in MDR. In addition to this, ABC proteins function as importer and exporter of certain molecules and nutrients from in and out of the cells and thus may present a major obstacle to anti-cancer therapies (Becker & Levy 2017). Among all the members of ABC transporter family associated with MDR, P-glycoprotein (P-gp) plays a major role in multidrug resistance. This P-gp is a product of ABCB1 (MDR1) gene and it has been documented that it provides resistance against antineoplastic agents. The high expression of P-gp in dysplastic cells or CSCs may augment drug resistance 100 times higher as compared to a normal cell (Breier et al. 2013). It has already been well reported that CD44 P-gp co-overexpression is associated with high progression of tumour with increased drug resistance in different tumors including ovarian cancer (Yang et al. 2015).

Based on the facts discussed, MDR in CSCs may lead to poor prognosis and low survival rates and has proven to be a challenging task in formulating effective strategies in treating all types of cancers including HNSCC (Govindan et al. 2015). Therefore, the goal of the current study was to describe and make it easier to comprehend how MDR1 expression and a marker for cancer cell stemness (CD44) relates to HNSCC.

Materials and Methods

Specimens

We collected tissue samples (incisional & excisional biopsies) of 66 cases of HNSCC who had undergone diagnostic or surgical resection for the disease in various maxillofacial surgical centers of Punjab Province, Pakistan. Each study participant gave their written informed permission. All tissue samples were delivered to the Department of Immunology UHS, Lahore in a suitable quantity of 10% formol saline, where they were given unique laboratory numbers and processed further.

Clinical details

We recorded information related to social, demographic history, habits, clinical signs, and symptoms of study subjects. Other relevant information regarding laboratory reports, radiography and surgical findings were obtained from patients' data files. All the information was collected on a specially designed proforma and recorded.

Gross examination

Detailed gross examination of each specimen was carried out and findings were recorded in the proforma.

Laboratory procedures

Hematoxylin & Eosin staining HNSCC tissue samples

After gross examination, representative tissue sections from intra-tumoural, marginal, para-tumoural, and distant normal tissues where ever possible were taken. An automated histology tissue processor was used for the processing of the biopsy specimen. Paraffin-embedded tissue blocks were made. At least three tissue sections of 4-6 µm thickness were cut by rotary microtome from each block which was then stained with haematoxylin and eosin to determine the diagnosis of squamous cell carcinoma and its histological grading/subtyping. Anneroth's System for histological grading was followed for grading of HNSCC (Akhter et al. 2011). After the diagnosis, the samples were processed for immunohistochemistry.

Immunohistochemistry of HNSCC tissue samples

Positively charged glass slides coated with poly-L-lysine were used for IHC and 3-5 μm thick tissue sections were mounted on these glass slides. After the deparaffinization step in a dry heat oven and xylene, these tissue sections' were rehydrated in graded ethyl alcohol with decreasing concentrations. Then, tissue sections passed through a heat-induced antigen retrieval step by immersing in citrate buffer solution (pH 6.0) in a water bath, and were put in the microwave oven before

staining. For immunostaining, Universal Kit (Lab Vision, USA) employing the streptavidin-biotin system was used to carry out the peroxidase-anti peroxidase method of immunohistochemistry staining. Sections were then incubated with primary monoclonal anti-CD44 antibody (A1078-100; BioVision, USA) and anti-P Glycoprotein antibody (EPR10364-57; ABCAM, USA). After incubation with primary antibodies, diaminobenzoate (DAB) chromogen was applied to the sections followed by counterstaining with haematoxylin for nuclear staining.

CD44 & MDR1/ABCB-1 Immuno-scoring

A semi-quantitative scale was adopted to measure the immunoreactivity score (IRS) for HNSCC tissue sections stained with antibodies described above. IRS was calculated by multiplying the staining intensity (SI) of tumor cells (0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining) by a proportion score (PC) based on the percentage of positive tumor cells (0=negative, 1<25%, 2=26%-50%, 3=51%-80%, 4 = 81% -100%). Results for PC and SI were multiplied, resulting in an immunoreactivity score (IRS) 0-12. The IRS was graded as high if the score were between 9 -12 and low for score ranging from 0 -8 (Boxberg et al. 2018).

FLOW CYTOMETRY ANALYSIS

To sort tumour cells with CD44 expression and enumeration of CSCs ($CD44^+$) for determining the expression of ABCB1/P-gp/MDR1 using flow-cytometry, single cell suspension was prepared from FFPET sections of HNSCC according to protocol described by Hedley et al. (1983). Flowcytometric analysis was carried out using the FACS Melody (BD Biotechnologies).

The cells were suspended in phosphate-buffered saline (PBS) and stained using anti-$CD44^-$FITC (BioLegend, San Diego, USA) and anti-MDR1-PerCP (BioLegend, San Diego, USA) antibodies. Anti-CD326-PE (abcam, MA, USA) was also added in the tubes to sort/gate the cells of epithelial origin. Two 5 mL tubes were labeled for each sample i.e., one for the test and the other for isotype control. The whole single cell suspension sample contained in 15 mL falcon tube was gently mixed by inversion and 500 µL sample was dispensed in each tube and then 3 µL of each antibody/Isotype controls were added in test and control tubes, respectively. The tubes were then vortexed and incubated at 4 °C in the dark for 10 min. Samples were centrifuged at 500 g for 5 min and the supernatant was discarded. The pellet was washed using 2 mL of 1X PBS. The samples were vortexed and centrifuged again at 500 g for 5 min. The pellet was re-suspended in 500 µL of 1X sheath fluid.

For flow cytometric analysis, results were acquired and analyzed with CellQuest Pro software. For each sample, 10000 events were acquired. Forward and side scatters were used to gate the epithelial cell population which was analyzed for cancer stem cell ($CD44^+$) and multidrug resistance markers (ABCB1/P-gp/$MDR1^+$).

STATISTICAL ANALYSIS

The numeric data were assessed as mean ±SD, median and interquartile ranges. To calculate the difference in mean expression of flow cytometric markers among different histological grades and subtypes of HNSCC, One-Way ANOVA, post hoc Tukey's, and Kruskal-Wallis tests were used. For each analysis, a p-value of ≤ 0.05 was considered as statistically significant. All the statistical analyses were performed using SPSS version 25.0 (IBM SPSS, Inc., Armonk, NY, USA) and Microsoft Excel for Windows.

ETHICAL CONSIDERATIONS

The ethical and technical approval to perform this study was granted by the Ethical Review Committee and the Advanced Studies & Research Board (No. UHS/Edu/126-16/1226) University of Health Sciences, Lahore, Pakistan. The Helsinki Declaration of the World Medical Association (WMA) was strictly adhered to by all researchers. The research study has guaranteed that none of the investigation study's findings and analyses will adversely affect anyone's features or values in any way.

RESULTS

Following Anneroth's system of histological grading for HNSCC, 66 cases in total were histologically examined on hematoxylin and eosin staining. Table 1 summarises the clinical and light microscopic characteristics of HNSCC cases. Among these 66 cases of HNSCC, 17 were categorized as WDSCC and 18 PDSCC. The dominant histological grade of HNSCC was MDSCC i.e., 31 (47%). On histological subtyping of HNSCC conventional type was 60 (90.9%), verrucous SCC 03 (4.5%), basaloid SCC 01 (1.5%) and basosquamous SCC 02 (3%) .

Immunohistochemical staining showed 52 (78.8%) cases had low/negative expression of MDR1/ABCB-1 antibody whereas high expression was observed in 14 (21.2%) cases. CD44; a marker of cell stemness was expressed highly in 39 (59.1%) cases of HNSCC while it was low in 27 (40.9%) cases (Figure 1).

TABLE 1. Clinical & light microscopic characteristics of HNSCC cases

	Study variables	Value n (%)
Age	(MeanS.D)	49.64±11.55
Gender	Male	40 (60.6)
	Female	26 (39.4)
Site of the tumour	Oral Cavity & Lip	52 (78.78)
	Oro-Pharynx	08 (12.12)
	Larynx	04 (6.06)
	Skin of face	02 (3.03)
Histological Grade	WDSCC	17 (25.8)
	MDSCC	31 (47)
	PDSCC	18 (27.27)
Histological Type	Conventional SCC	60 (90.9)
	Verrucous SCC	3 (4.5)
	Basaloid SCC	1 (1.5)
	Basosquamous SCC	2 (3)
MDR-1 IRS	Low/Negative expression	52 (78.8)
	High expression	14 (21.2)
CD44 IRS	Low/Negative expression	27 (40.9)
	High expression	39 (59.1)

SCC Squamous Cell Carcinoma, WD Well Differentiated, MD Moderately Differentiated, PD Poorly Differentiated

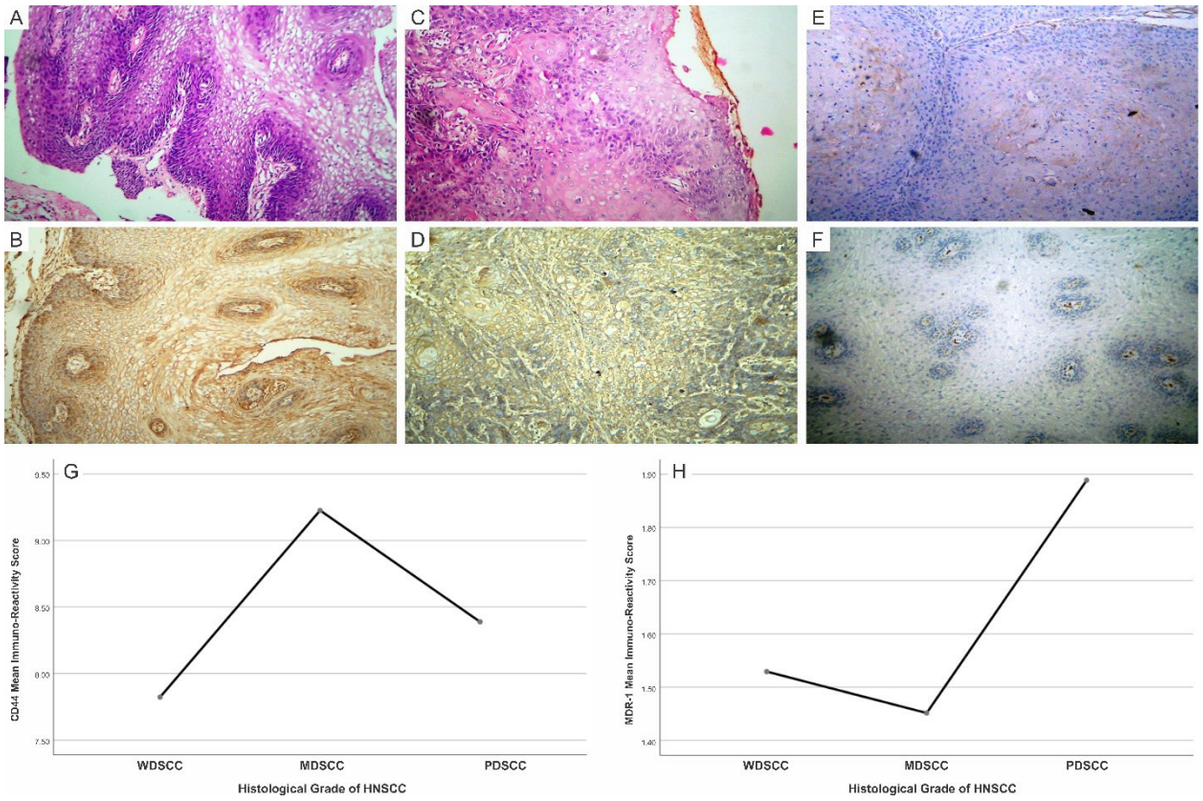

FIGURE 1. Histological grading and IHC staining of HNSCC: A Grade I HNSCC (Verrucous subtype) with multiple epithelial finger like projections (H&E 100X); B Characteristics membranous staining and moderate positivity with CD44 (IHC 100X); C Grade II, moderately differentiated HNSCC (H&E 100X); D CD44 antibody stained grade II HNSCC showing membranous staining with moderate intensity (IHC 100X); E & F Poorly differentiated HNSCC with low intensity of CD44 & MDR1 staining; G & H Graphical presentation of CD44 and MDR1 IRS in different histological grades of HNSCC

The mean IRS for CD44 was 8.63 ±3.02. For MDR1/ABCB-1 antibody staining, the mean was 1.59 ±1.27. In the comparison of immune-expression scores of CD44 antibody and MDR1/ABCB-1 the relationship was not statistically significant (Table 2).

Histological grades were compared with the immune-reactivity status of CD44 and MDR1/ABCB-1 antibodies in HNSCC cases. The description of positivity and negativity for both of the antibodies has been already described above in the 'Materials and Methods' section. The expression of both antibodies was found to be statistically insignificant in different grades of HNSCC (Tables 3 and 4).

A total of 10000 events were acquired from single cell suspension of each FFPET sample prepared for flow cytometric analysis. Only the cells with strong expression of CD326-PE were gated and further analyzed for the determination of different subsets of squamous epithelial tumour cells showing the expression of CD44-FITC and MDR1-PerCP (Figure 2(A), 2(B), 2(C)).

TABLE 2. Mean immune-reactivity score of CD44 and MDR1/ABCB1 in HNSCC cases (IHC)

Variables	N	Minimum	Maximum	Mean	Std. Deviation
CD44 immuno-reactivity score	66	2.00	12.00	8.63	3.02
MDR-1 immuno-reactivity score	66	0.00	6.00	1.59	1.27

TABLE 3. Comparison of histological grade and CD44 immuno-reactivity

Variables		CD44 immuno-reactivity status		Total
		Low/Negative expression	High expression	
Histological Grade of HNSCC	WDSCC	9	8	17
	MDSCC	10	21	31
	PDSCC	8	10	18
Total		27	39	66

Fisher's Exact p=0.420

TABLE 4. Comparison of histological grade and MDR-1/ABCB-1 positivity status

Variables		MDR-1 positivity status		Total
		Low/Negative expression	High expression	
Histological grade of HNSCC	WDSCC	14	3	17
	MDSCC	25	6	31
	PDSCC	12	6	18
Total		51	15	66

Fisher's Exact p=0.789

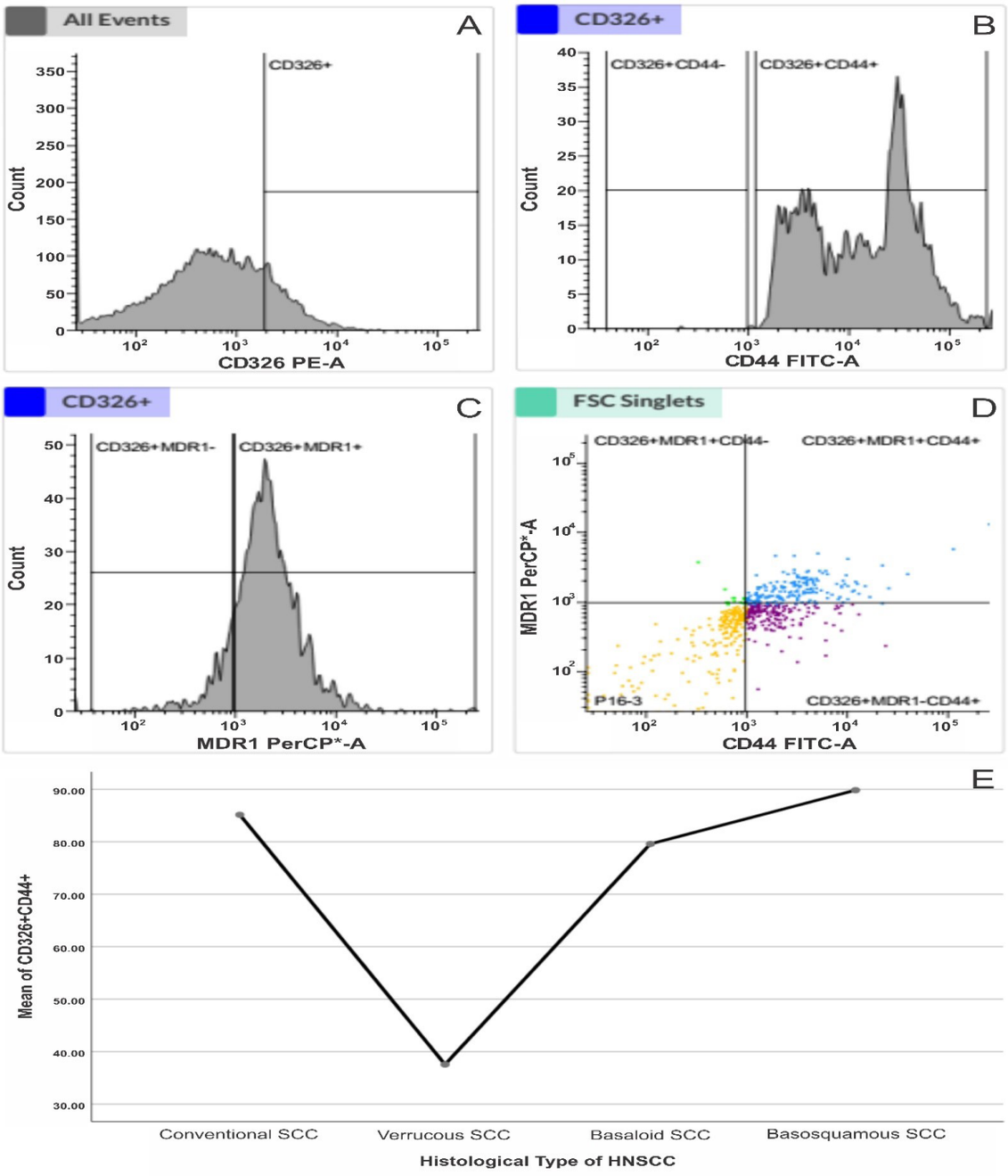

FIGURE 2. The dot plots and line graphs of representative data demonstrate the analysis protocol for the identification of different subsets of tumorous squamous epithelial cells in single suspension prepared from FFPET section of HNSCC following three color-staining. A Tumour cell subset was gated based on expression of CD326; B Then another subset $CD326^{+}CD44^{+}$ (Possibly CSCs) was also identified on dot plot. C In the next dot plot, another subset of tumour cells showing the marker of multidrug resistance was gated and marked. D Final dot plot with forward scattering-Singlets is showing a subset of tumour cells characterized by positivity for $CD326^{+}CD44^{+}MDR1^{+}$. E Line graph illustrating the mean score of $CD326^{+}CD44^{+}$ cell subsets in various histological subtypes of HNSCC

When compared to dysplastic tumour cells with characteristics of cell stemness ($CD326^+CD44^+$, 83.03±19.09), the mean score for the fraction of tumour cells with cell stemness and drug resistance ($CD326^+CD44^+MDR1^+$) was fairly low (16.28±15.60). Therefore, it can be concluded that a relatively small population of HNSCC tumour cells is capable of medication resistance. The mean numbers of different subsets of tumorous squamous epithelial cells were also measured among different grades and histological subtypes of HNSCC. When one-way ANOVA was applied to compare the mean score of both subsets of tumour cells ($CD326^+CD44^+$ & $CD326^+CD44^+MDR1^+$) among various histological grades of HNSCC, an insignificant statistical difference was found (Table 5). Hence, it can be concluded that histological grade has no role in determining the tumour progression, recurrence and resistance to antineoplastic therapy.

When the mean score of the subset of tumour cells with hallmarks of cell stemness ($CD326^+CD44^+$, most probable/documented CSCs subset) was compared among the various histological subtypes of HNSCC, it was interesting to note that a significant statistical difference ($p = 0.000$) was found. While the mean scores for subpopulation of cells positive for both markers ($CD44^+MDR1^+$) among different histological subtypes were not found to be statistically significant (Table 6). Therefore, it was observed that in patients with HNSCC, the tumour behaviour could be determined by the histological subtype rather than the histological grade.

TABLE 5. Comparison of different subsets of tumour cells with histological grades of HNSCC

Subsets of tumour cells	Histological grade	N	Mean (%)	Std. Deviation	One-way ANOVA *p* Value
$CD326^+CD44^+$ Dysplastic epithelial cells with cell stemness potential	WDSCC	17	85.0312	17.08167	0.846
	MDSCC	31	82.9977	22.28834	
	PDSCC	18	81.2183	15.79234	
	Total	66	83.0362	19.19078	
$CD326^+CD44^+MDR1^+$ Dysplastic epithelial cells with cell stemness potential & drug resistance	WDSCC	17	19.0500	22.15001	0.552
	MDSCC	31	16.5123	14.53307	
	PDSCC	18	13.2600	8.91738	
	Total	66	16.2789	15.60788	

DISCUSSION

HNSCCs remain a significant cause of morbidity and mortality and have been proved as a major public health burden all across the world even though their early diagnosis and treatment have been made possible due to modern-day advancements. Gupta, Johnson and Kumar (2016) have speculated a shift of HNSCCs burden from developed to developing countries and these less developed and ill-equipped countries may not be able to cope with this increasing burden. Therefore, policymakers and cancer organizations should plan and implement population-based interventions urgently to control HNSCCs burden.

In the current study, Anneroth's System of Histological Grading for HNSCC was followed and the majority of cases were MDSCC 31 (47%), followed by WDSCC and PDSCC. A study carried out in South Korea in 2011, reported that among 54 cases of HNSCC,

TABLE 6. Comparison of different subsets of tumour cells with histological subtypes of HNSCC

Subsets of tumour cells	Histological subtypes	N	Mean (%)	Std. Deviation	One-Way ANOVA *p* value
$CD326^{+}CD44^{+}$ Dysplastic epithelial cells with cell stemness potential	Conventional SCC	60	85.1423	16.73735	0.000
	Verrucous SCC	3	37.5400	18.88803	
	Basaloid SCC	1	79.5800	.	
	Basosquamous SCC	2	89.8250	10.81166	
	Total	66	83.0362	19.19078	
$CD326^{+}MDR1^{+}CD44^{+}$ Dysplastic epithelial cells with cell stemness potential & drug resistance	Conventional SCC	60	15.2770	13.67246	0.264
	Verrucous SCC	3	31.6967	40.97335	
	Basaloid SCC	1	11.3000	.	
	Basosquamous SCC	2	25.7000	21.72232	
	Total	66	16.2789	15.60788	

34 (62.96%) were WDSCC, 10 (18.5%) as MDSCC, and 10 (18.5%) as PDSCC (Zou et al. 2017). The findings of this study were quite contrary to the results of the present study because WDSCC cases were predominant in this study. A study carried out in Pakistan reported that moderately differentiated tumors were observed (51.9%) (Minhas et al. 2016). Even our findings were different previously, with WDSCC reportedly higher in numbers (Kashif et al. 2015). Actually, tumour epidemiology and heterogeneity are mainly a result of social factors such as addictive and sexual habits. The tumour grades are perhaps associated with genetic or epigenetic alterations between different tumor cell types.

Nearly half a century ago, cancer researchers proposed a cancer stem cell theory and this theory states that a subpopulation of cells with properties of stemness is at the crest of hierarchy in a pool of cancer cells and these CSCs not only symmetrically split and complement the CSC pool but, also provide low tumourigenic/dysplastic cells by further asymmetric division. One very interesting and important fact that should be mentioned here is that like normal stem cells of the body, CSCs also demonstrate the enhanced potential for self-renewal, proliferation, and differentiation. The studies conducted so far have established that $CD44^{+}$ cells display the characteristics of CSCs in HNSCC (Chen & Wang 2019). As described earlier, in the present study, 39 (59.1%) of HNSCC cases were strongly positive for CD44 antibody on IHC staining but in comparison, this high CD44 expression was not associated significantly with histological grades of the HNSCC. The findings of the study carried out by Chen et al. (2014) were also in line with the current study, they reported that in their study cases of HNSCC, the total mean percentage of CD44 expression was 57.8%, with a gradual increase in expression in SCC of the oral cavity, pharynx and larynx i.e., 49.3%, 66.4%, 54.7%, respectively. Their findings demonstrated no significant correlation between clinical features and expression of CD44 in cases of OSCC but other subcategories of HNSCC showed a significant association between expression of CD44, tumour size (T), and nodal involvement (N) according to TNM staging system. Another study performed at Stanford University Medical Centre, California by Joshua et al. (2012) reported that on flowcytometry, the mean frequency of

CD44$^+$ lineage was 25%. Although expression was low as compared to Chen's and the current study, it was correlated with poor prognostic factors i.e., advanced tumour stage and high recurrence rate.

A very minute subpopulation of cancer cells positive for CD44 show properties of cell stemness as well as increased expression of cell surface drug efflux pump proteins i.e., P-gp. Among many discovered members of ABC transporter family, this P-gp is an ABCB1 member. A very high and massive expression of P-gp in CSCs/CD44$^+$ cells can induce 100 times more drug resistance capacity to anti-cancer drugs. It has been documented by many researchers that P-gp is a real obstacle to chemotherapeutic agents and effectively treating malignant diseases. Therefore, this protein should be considered a viable target for pharmaceutical design (Becker & Levy 2017; Yang et al. 2015). Only 14 (21.21%) cases of HNSCC in the current study showed medium to high expression of MDR1/ABCB-1 antibody and were considered positive on a semi-quantitative scale as described. In an animal model study, Yang et al. (2015) observed that on administering the siRNA targeting against MDR1 and CD44, the expression of both genes and their proteins decreased in MDR cells. The findings of that *in vivo* study demonstrated that average tumour volume could be reduced by targeting MDR1 and CD44 genes using siRNA, nanoparticles, and a potent anticancer drug (pacilatxel) as compared to the control group. Several previously published studies have reported that high P-gp expression could lead to multidrug resistance in HNSCC, but a mechanism that incites this high expression of P-gp is still unclear and needs to be researched (Brock et al. 1995; Chaudhary & Roninson 1993).

Shen et al. (2013) reported that CD44$^+$ tumour cells in hypopharyngeal cancer cell lines had a stronger proliferative potential and more tumourigenic capacity when injected in mice as compared to CD44$^-$ tumour cells with a statistically significant difference between the two groups. They concluded that CD44 is a marker of aggressive tumour behavior. This finding is quite consistent with our finding of a strong association (p = .000) of CD44$^+$ tumour cells in basaloid squamous and baso-squamous variants of HNSCC which are aggressive tumour subtypes. On contrary to this, Vikram et al. (2020) reported that CD44$^-$CD24$^-$ cell population in breast cancer cell lines possess more tumorigenic and metastatic potential. They were of the opinion that CD44 expression does not correlate with tumorigenic potential and cells devoid of CD44 expression also contains properties of progenitor/stem cells.

Conclusion

CD44 and ABCB-1/MDR1 are both present in HNSCC tissue samples, but CD44 shows higher expression levels on a semi-quantitative scale and is more prominently detected through immunohistochemistry. However, despite their co-expression, no significant correlation was observed between the various grades and subtypes of HNSCC and the expression of these markers. While on flowcytometric analysis of a single cell suspension of tumorous squamous cells prepared from FFPET sections of HNSCC, a strong statistical difference (p = 0.000) had been observed when the mean score of subset of dysplastic cells with characteristics of cell stemness (CD326$^+$CD44$^+$, most probable/documented CSCs) was compared among different histological subtypes of HNSCC. Although single cell suspension prepared from FFPET section of HNSCC could be potential source of research but further studies should be performed on primary cell culture prepared from vital tissue samples of HNSCC to attain more precise and definitive findings.

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