


Autophagy regulates cisplatin-induced stemness and chemoresistance via the upregulation of CD44, ABCB1 and ADAM17 in oral squamous cell carcinoma

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Abstract

Objective: We inspected the relevance of CD44, ABCB1 and ADAM17 in OSCC stemness and deciphered the role of autophagy/mitophagy in regulating stemness and chemoresistance.

Material and methods: A retrospective analysis of CD44, ABCB1 and ADAM17 with respect to the various clinico-pathological factors and their correlation was analysed in sixty OSCC samples. Furthermore, the stemness and chemoresistance were studied in resistant oral cancer cells using sphere formation assay, flow cytometry and fluorescence microscopy. The role of autophagy/mitophagy was investigated by transient transfection of siATG14, GFP-LC3, tF-LC3, mKeima-Red-Mito7 and Western blot analysis of autophagic and mitochondrial proteins.

Results: In OSCC, high CD44, ABCB1 and ADAM17 expressions were correlated with higher tumour grades and poor differentiation and show significant correlation in their co-expression. In vitro and OSCC tissue double labelling confirmed that CD44⁺ cells co-expresses ABCB1 and ADAM17. Further, cisplatin (CDDP)-resistant FaDu cells displayed stem-like features and higher CD44, ABCB1 and ADAM17 expression. Higher autophagic flux and mitophagy were observed in resistant FaDu cells as compared to parental cells, and inhibition of autophagy led to the decrease in stemness, restoration of mitochondrial proteins and reduced expression of CD44, ABCB1 and ADAM17.

Conclusion: The CD44⁺/ABCB1⁺/ADAM17⁺ expression in OSCC is associated with stemness and chemoresistance. Further, this study highlights the involvement of mitophagy in chemoresistance and autophagic regulation of stemness in OSCC.

1 | INTRODUCTION

Regardless of the development in early detection, diagnosis and multimodal treatment strategies, oral squamous cell carcinomas (OSCC) is often associated with a high rate of locoregional recurrence, ipsilateral and bilateral lymph node metastasis and chemo- and radioresistance.¹

The marginal improvement in the overall 5-year survival rate of the OSCC patients and the inefficacy of the cotemporary adjuvant chemoradio-therapeutics is now been ostensible to the arbitrary cytoreductive approaches that are designed to target bulk tumour cells setting aside a small subset of resistant tumour cells which could propagate the tumour post-treatment. This resistant subpopulation of cells with

exclusive self-renewal capacity, hierarchical differentiation and tumorigenicity is referred as Cancer Stem Cells (CSCs).²

Autophagy is an evolutionary conserved lysosome-dependent self-digestive recycling mechanism for providing metabolite for biosynthesis and energy synthesis as a response to stress conditions. Although autophagy functions as a tumour suppressor mechanism, it also shows protumour activity to facilitate cell survival during cancer progression.² Substantial studies suggest the significant contribution of autophagy in the resistance of tumour cells to chemotherapy.³ Moreover, increased autophagic flux is also reported to induce resistance against therapy-induced cytotoxicity via maintaining the survival of CSCs through conferring stress tolerance and limiting damages.² Recently, the concept selective autophagy came forward which emphasizes role of organellar autophagy like selective mitochondrial autophagy or "Mitophagy" in the context of cancer. The failure to properly modulate mitochondrial quality control via mitophagy in response to oncogenic stresses might regulate the tumorigenesis both positively and negatively.^{4,5} Furthermore, chemoresistant cancer cells are reported to exhibit stem cell-like features.⁶ This study elucidates the role of autophagy in inducing stemness and chemoresistance in oral cancer.

CD44, most commonly studied CSC surface marker in oral cancer, is a transmembrane receptor which binds with hyaluronic acid (HA) and signals many cancer-related events such as proliferation, invasion and metastasis. CD44 is also associated with apoptosis resistance, drug resistance and stemness.⁷ The ABCB1 subfamily of ABC transporters or the P-glycoproteins responsible for reduced intracellular accumulation of chemotherapeutics is highly expressed in CSCs. But the expressions of ABCB1 in oral cancer are not well studied.⁸ ADAM17 (A disintegrin and metalloproteinase) or TACE (TNF-alpha converting enzyme), a membrane protein responsible for the cleavage CD44 ectodomain, extracellular matrix remodelling, invasion and metastasis of cancer, is also associated with inducing stemness and driving tumorigenesis in head and neck cancer.⁹ The purpose of this study is to investigate the clinical significance of CD44⁺/ABCB1⁺/ADAM17⁺ cells in resected tumour specimens of OSCC using the immunohistochemical assay and highlight the relevance of the expression profile of CD44⁺/ABCB1⁺/ADAM17⁺ in context of stem cell hypothesis, autophagic regulation of stemness and chemoresistance in OSCC. More importantly, this study emphasizes that selective autophagy like mitophagy might be responsible for chemoresistance in cancer.

2 | MATERIALS AND METHODS

2.1 | Patients and tumour specimen

Archival formalin-fixed and paraffin-embedded (FFPE) tumour specimens from sixty cases of OSCC were collected from Drs. Tribedi and Roy Diagnostic Laboratory, Kolkata, local hospitals, nursing homes and clinics near the Ranchi area and Bangalore, India. The material had been stored with permission of the local ethics committee after informed consent obtained from the patients prior to surgical resection. Tumour and patient characteristics are summarized in Table S1. The tumour blocks of paraffin-embedded tissue were selected by

experienced pathologists, evaluating the routine H.E.-stained sections. Sections from all available tumours underwent intensive histopathologic assessment, blinded to the prior histopathology report. Serial tissue sections (5 µm thickness) were cut from FFPE blocks on a microtome and mounted from warm water onto adhesive microscope slides. Tumour Grading was performed according to WHO criteria. Tumour characteristics and patient characteristics were collected in a database (Excel, Microsoft) for further analysis.

2.2 | Immunohistochemistry

2.2.1 | Immunostaining

For immunohistochemical analysis, unconjugated specific ABCB1 (1:100, abcam # ab155421), ADAM17 (1:100, abcam # ab574821) (Abcam, Inc., Cambridge, UK) and CD44 antibody (1:100, Immunotools # 21810441) were used. Pretreatment and immunohistochemical single staining procedure were performed as described in the Super Sensitive™ Polymer HRP IHC Detection System procured from Biogenex, Fremont, CA, USA.

2.2.2 | Assessment standard

Each stained tumour specimen was photographed for 6 randomly selected fields with Olympus IX71 microscope, and all assessment were made at a magnification of ×200. The average of their evaluations was calculated for statistical analysis. Each slide was evaluated independently by 2 independent observers who did not know the clinical outcomes. The expression of each antibody was evaluated for each tissue sample by calculating the total Immuno-reactive Score (IRS) as the product of the proportion and intensity scores. The proportion score reflected the estimated fraction of positive-stained tumour cells (0, none; 1, 1%-25%; 2, 26%-50%; 3, 51%-75%; 4, 76%-100%). The intensity score represented the estimated staining intensity (0, no staining; 1, weak/light yellow; 2, moderate/buffy; 3, strong/brown).¹⁰ The total IRS ranged from 0 to 12, and the scores were averaged. Scores of 0-5 were designated as low expression, while scores of 6-12 were designated as high expression. Moreover, IRS scores: 0 is considered (-); 1 to 4 (+); 5 to 8 (++); and 9 to 12 (+++).

2.3 | Cell culture

The human hypopharyngeal cancer cell line FaDu was procured from American Type Culture Collection ([ATCC], Manassas, USA). FaDu cells were cultured in MEM complemented with 10% fetal bovine serum and antibiotic-antimycotic solution followed by incubation at 37°C in a humidified (95% air: 5% CO₂) incubator.

2.4 | Establishment of CDDP resistance in OSCC cell line

FaDu cells were repeatedly subcultured in MEM containing increasing concentrations *cis*-Diammineplatinum (II) dichloride (CDDP/cisplatin) (Sigma-Aldrich, St. Louis, MO, USA) over a 3-month period. FaDu cells

that grew in 0.5 μM CDDP were designated as FaDu CDDP resistant (FaDu CDDP-R) and maintained in MEM containing 0.5 μM cisplatin, whereas the parental FaDu cells were designated as FaDu-P.

2.5 | Drug sensitivity assay

FaDu-P and FaDu-CDDP-R cell concentration was adjusted to 5×10^4 cells/mL, plated in 96-well flat-bottom culture plates and incubated with various concentrations of CDDP for 72 h. The effect of CDDP on cancer cell viability was studied using MTT dye reduction assay by measuring the optical density at 595 nm using a micro-plate reader spectrophotometer (Perkin- Elmer, Waltham, MA, USA).

2.6 | Sphere formation assay

FaDu-P and FaDu-CDDP-R cells were cultured in serum-free medium supplemented with 2% B27 supplement (Gibco, Gaithersburg, MD 20877, USA), 1% N2 supplement (Gibco, Gaithersburg, MD 20877, USA), 20 ng/mL epidermal growth factor (EGF; BD Biosciences, Franklin Lakes, NJ, USA) and 20 ng/mL basic fibroblast growth factor-2 (bFGF-2; BD Biosciences Franklin Lakes, NJ, USA) and maintained in ultralow attachment plates (Corning, NY 14831, USA) at 37°C in a humidified 5% CO₂ incubator. After 10 days of culture, the number of non-adherent spherical clusters of cells or oospheres was counted under a microscope.

2.7 | Immunocytofluorescence staining and analysis

FaDu-P and FaDu-CDDP-R cells were fixed with 10% formaldehyde, permeabilized by 0.1% Triton X-100, and then incubated with the primary antibodies for CD44 (1:500, Immunotools # 21810441), ABCB1 (1:500, abcam # ab155421), ADAM17 (1:500, abcam # ab574821), β -catenin (1:500, BD Biosciences # 610153), LC3B (1:500; Novus Biologicals # NB-100-2220), TOM20 (1:500, BD Biosciences # 612278). The secondary anti-rabbit and/or anti-mouse antibodies conjugated with Alexa Fluor (1: 500; A11001, A11004, A11011 and A11008) from Invitrogen were used to study the fluorescence of our desired proteins, which were detected using an Olympus IX71 fluorescent inverted microscope and the cellSens Standard software (version 1.6, Olympus Soft Imaging Solutions GmbH, Johann- Krane-Weg, Münster, Germany). Immunofluorescence double staining tissue for CD44, ABCB1 and ADAM17 were done as described in Grimm et al¹¹. The immunocytofluorescent intensities were quantified by measuring the integrated optical density and area fraction with the ImageJ software (National Institute of Mental Health, Bethesda, MA, USA).¹²

2.8 | Flow cytometry

The FaDu-P cells and FaDu-CDDP-R cells were harvested and incubated with PE-labelled CD44 antibodies (BD Biosciences # 550989) for 30 min at room temperature in dark. Then, the cells were washed with ice-cold PBS (10 mM, pH 7.4) and re-suspended in 500 μL of

PBS. The expression of CD44 and percentage of CD44⁺ population were analysed with the help of BD ACCURI C6 flow cytometer and FCS EXPRESS software.

2.9 | Western blot

Samples containing equal amounts of protein were subjected to SDS-PAGE and transferred onto PVDF membrane. The blots were incubated with primary antibody against LC3B (1:1000; Novus Biologicals # nb-100-2220), SQSTM1/p62 (1:1000, BD Biosciences # 610832), ULK1 (1:1000, Cell Signaling and Technology # 80545), Beclin 1 (1:1000, Cell Signaling and Technology # 3738), Atg5 (1:1000, Cell Signaling and Technology # 2630S), Atg7 (1:1000 Biogenesis-R-161-100), ATG14 (1:1000, Cell Signaling and Technology # 5504BC), COX-IV (1:1000, Cell Signaling and Technology # 4844S), actin (1:1000, Sigma-Aldrich # A2066) followed to peroxidase labelled anti-mouse (1:1000, Cell Signaling and Technology # 7076)/anti-rabbit (1:1000, Cell Signaling and Technology # 7074) secondary antibodies. The Western blot band intensities were quantified by measuring the integrated optical density and area fraction with the ImageJ software (National Institute of Mental Health).¹²

2.10 | Small-interfering RNA and plasmid transfection

The indicated FaDu cells were transfected with siRNA for ATG14 by using Lipofectamine 2000 reagent following the manufacturer's instructions. Control-siRNA was used as a negative control. The tandem GFP-RFP-LC3 transfection was performed to study the autophagic flux in parental and resistance FaDu cells, and mKeima-Red-Mito7plasmid transfection was carried out to investigate the lysosomal delivery of mitochondria to be degraded by autophagy. A corresponding backbone plasmid was used as negative control for each plasmid transfection.

2.11 | Statistical analysis

The data are presented as the mean \pm SD. Statistical comparisons between subgroups were made using the appropriate statistical test (chi-square, chi-square for linear trend tests and Fisher exact test). The correlation between the marker proteins was analysed by Spearman rank test. Student's *t* test was used for evaluating statistical differences between experimental groups. The analysis was performed by GraphPad Prism 4.0 software. A 2-tailed *P*-value test was used in all analyses, and the *P* value was defined as follows: not significant (n.s.): $P > 0.05$; *: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$; ****: $P \leq 0.0001$ were considered statistically significant.

3 | RESULTS

3.1 | Clinical presentation of disease in this cohort

Numerous clinical characteristics of our cohort are described in Table S1. The median age of the patients was 51 years. Out of 60

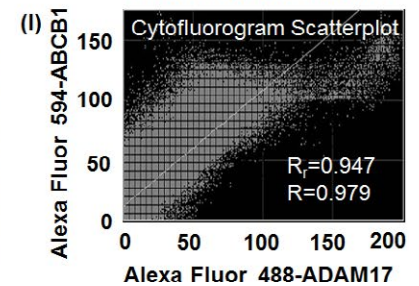
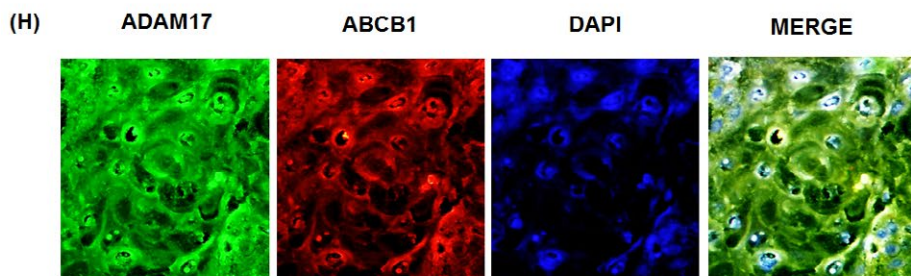
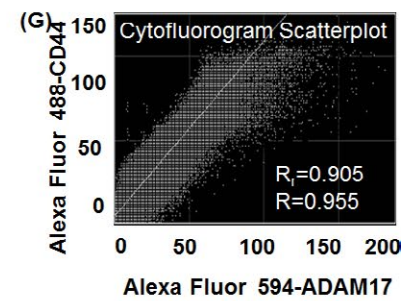
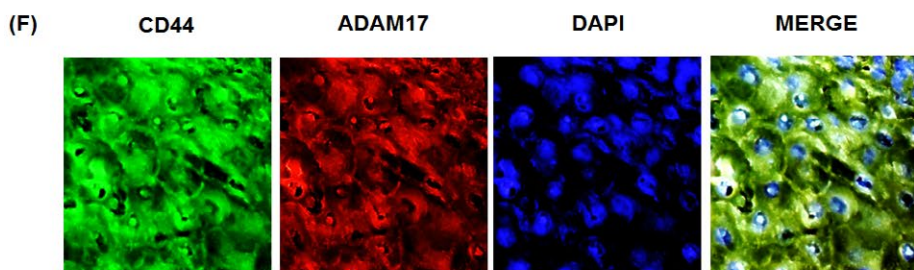
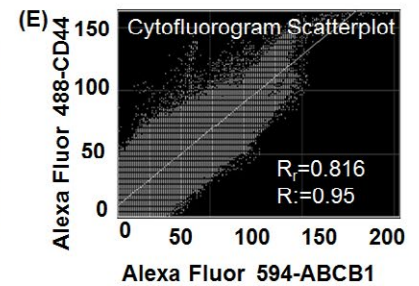
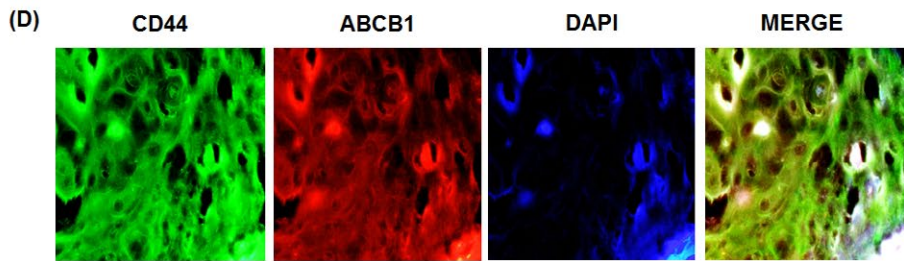
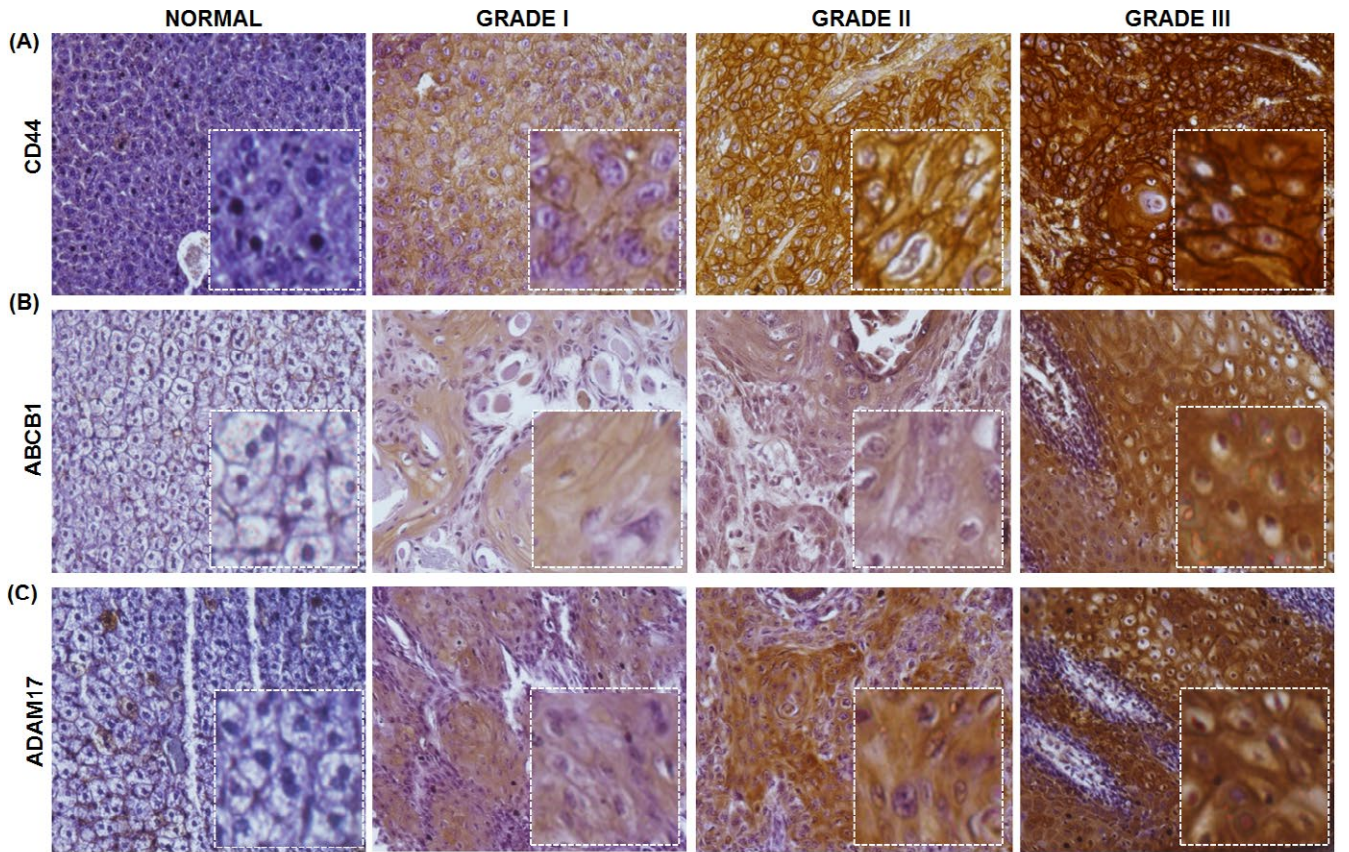


FIGURE 1 Expression of CD44, ABCB1 and ADAM17 in normal oral tissue and oral squamous cell carcinoma tissue and their co-expression. Slide shows representative images of CD44 (A), ABCB1 (B) and ADAM17 (C) staining in normal oral tissue and different grades of OSCC tissue samples. Brown chromogen colour (3,3-Diaminobenzidine) indicates positive CD44, ABCB1 and ADAM17 staining and the purple colour indicates the nuclear counterstaining by haematoxylin. The square box demonstrates the area of interest shown in larger magnification. Images demonstrate a representative immunofluorescent double labelling of indicated proteins and their cytofluorogram scatter plot depicting the co-expression (D-I)

cases, 27 (45%) patients were ≤ 51 years, 22 (36.6%) patients were above 51 years, whereas the age details of 11 (18.33%) patients were not available. Our cohort included 30 (50%) male patients and 25 (41.66%) female patients and the gender details of 5 (8.33%) cases were not available. With respect to the histological characteristics and grading of the cancers showed that within our cohort, 32 (53.33%) patients had Grade I (well differentiated) OSCC, 19 (31.66%) had Grade II (moderately differentiated) and 9 (15%) patients had Grade III (poorly differentiated) tumours. We concluded that this cohort was suitable for determining the relative usefulness of CD44, ABCB1 and ADAM17 expression as an indicator in CSC hypothesis and chemoresistance.

3.2 | CD44, ABCB1 and ADAM17 expressions and their association with clinico-pathological features of OSCC

Expression of CD44 was mostly membranous, and the positive expression rate of CD44 in OSCC was 90% (54/60). The IRS of cancer cells with CD44 expression ranged from 0 to 12 (mean 7.73, median 8). The 85% (51/60) cases were considered positive for the expression of ABCB1. The IRS of the cancer cells with ABCB1 expression ranged from 0 to 12 (mean 4.5, median 4). Likewise, 91.7% (55/60) cases were ADAM17 positive. The IRS of the cancer cells with ADAM17 expression ranged from 0 to 12 (mean 6.11, median 8) (Table S2). The expression of CD44 (Figure 1A), ABCB1 (Figure 1B) and ADAM17 (Figure 1C) apparently increased in a grade-wise manner in OSCC tissue samples. The expression profiles of CD44, ABCB1 and ADAM17 were similar in OSCC tissue. The high expression percentages of CD44, ABCB1 and ADAM17 in Grade I (well differentiated) OSCC tumours were 31.25% (19/32), 15.62% (5/32) and 40.62% (13/32), respectively, which were significantly lower than the Grade II (moderately differentiated) OSCC tumours: 78.94% (15/19), 52.63% (10/19) and 73.68% (14/19), respectively. The CD44, ABCB1 and ADAM17 high expression in Grade III (poorly differentiated) OSCC tumours were 100% (9/9), 88.88% (8/9) and 88.88% (8/9), respectively, which were considerably high than Grade I and Grade II OSCC tumours with all $P < 0.05$ ($P = 0.0129$, $P = 0.0001$, $P = 0.0026$). However, expression profiles of CD44, ABCB1 and ADAM17 did not associate with age and sex of the patients (P all > 0.05) (Table 1).

Next, we investigated whether there is any link between CD44, ABCB1 and ADAM17 with STRING 10.5 (<https://string-db.org/>) protein-protein interaction online software. Protein-protein interaction (PPI) enrichment P -value for CD44, ABCB1 and ADAM17 interaction is 0.00523, indicating that these proteins have more interactions among themselves than what would be expected for a random set of proteins of similar size, drawn from the genome (Figure S1). From the

statistical analysis, we found a positive correlation between CD44 (or ADAM17) and ABCB1, as well as between CD44 and ADAM17 in OSCC. The CD44 and ABCB1 share a moderate positive correlation (Spearman correlation coefficient or r_s : 0.574), whereas CD44 and ADAM17 and CD44 and ABCB1 share a weak positive relation with r_s 0.428 and 0.346, respectively, all $P < 0.01$) (Table S3). Furthermore, pairwise correlation analysis of these 3 proteins shows that the high expression of CD44/ABCB1, CD44/ADAM17 and ABCB1/ADAM17 was correlated with tumour grades and tumour differentiation (all $P < 0.05$). Also, the pairwise correlation analysis of these 3 proteins did not show a relationship with age and sex of the patients (all $P > 0.05$) (Table 2). Further, we analysed the correlation between the triple high expression (tissue samples having high expression status for all 3 proteins) and triple non-high expression (tissue sample having at least one low expression status among the 3 proteins). It is observed that triple high expression of CD44/ABCB1/ADAM17 was remarkably correlated with tumour grades and tumour differentiation (all $P < 0.05$). However, it did not show any relation with age and sex of the patient (all $P > 0.05$) (Table 3).

To evaluate the hypothesis that putative CD44⁺ CSC are associated with ABCB1 and ADAM17 expression, immunofluorescent double-labelling experiments were operated in OSCC tissue sections and cell lines. We found a dominant population of CD44⁺/ABCB1⁺ tumour cells (Figure 1D,E) with Pearson's coefficient of 0.816 and overlap coefficient of 0.95 and CD44⁺/ADAM17⁺ tumour cells with Pearson's coefficient of 0.905 and overlap coefficient of 0.955 (Figure 1F,G) in OSCC tissues indicating that CD44⁺ cells highly co-expresses ABCB1 and ADAM17. Moreover, we observed the co-expression of ABCB1 and ADAM17 in OSCC tissue samples (Figure 1H,I) with Pearson's coefficient of 0.947 and overlap coefficient of 0.979. Further, immunohistochemical double staining was reevaluated in FaDu cells and CD44⁺/ABCB1⁺ (Figure S2A,B) and CD 44⁺/ADAM17⁺ (Figure S2C,D) co-expressing population as well as ABCB1⁺/ADAM17⁺ co-expression in FaDu cell (Figure S2E,F) was observed.

3.3 | CDDP-resistant cells are bestowed with cancer stem-like features and increased expression of CD44, ABCB1 and ADAM17

Therapeutic resistance is a major concern encountered during the treatment of OSCC. To gain further insights into the mechanisms of chemoresistance and its correlation with stemness, we established the cisplatin (CDDP)-resistant FaDu cell lines (FaDu-CDDP-R). The parental FaDu (FaDu-P) cells were treated with incremental concentration of cisplatin ranging from 0.01 μM to a final concentration 0.5 μM for a period of 3 months to generate FaDu-CDDP-R cells. Once the resistant phenotype was established, the cells were maintained by continuous

TABLE 1 Relationship between CD44, ABCB1 and ADAM17 and the clinico-pathological features OSCC

Groups	Cases	CD44			ABCB1			ADAM17		
		Low	High	P value	Low	High	P value	Low	High	P value
Gender	55 ^a	–	–	0.5616	–	–	0.5946	–	–	0.7903
Male	30	8	22		19	11		12	18	
Female	25	9	16		14	11		11	14	
Age	49 ^b	–	–	0.1317	–	–	1.000	–	–	0.5675
≤51	26	12	14		17	9		13	13	
>51	23	5	18		15	8		9	14	
Tumour Grade	60	–	–	0.0129*	–	–	0.0001****	–	–	0.0026**
Grade I	32	13	19		27	5		19	13	
Grade II	19	4	15		9	10		5	14	
Grade III	9	0	9		1	8		1	8	

^aFive cases of OSCC patients without gender details.

^bEleven cases of OSCC patients without age details.

* $P \leq .05$; ** $P \leq .01$; **** $P \leq .0001$.

TABLE 2 Relationship between CD44/ABCB1, ABCB1/ADAM17 and CD44/ADAM17 expression and the clinico-pathological features of OSCC

Groups	Age in years ^a			Gender ^b			Tumour grades			
	≤51	>51	P value	Male	Female	P value	Grade I	Grade II	Grade III	P value
CD44/ABCB1										
CD44 ^{High} ABCB1 ^{High}	7	6	0.1769	11	8	0.7207	4	8	8	0.0007***
CD44 ^{High} ABCB1 ^{Low}	7	12		11	8		15	7	1	
CD44 ^{Low} ABCB1 ^{High}	2	1		0	3		1	2	0	
CD44 ^{Low} ABCB1 ^{Low}	10	4		8	6		12	2	0	
ABCB1/ADAM17										
ABCB1 ^{High} ADAM17 ^{High}	6	4	0.6950	9	6	0.7359	1	7	7	0.0001***
ABCB1 ^{High} ADAM17 ^{Low}	3	3		2	5		4	3	1	
ABCB1 ^{Low} ADAM17 ^{High}	7	10		9	8		12	7	1	
ABCB1 ^{Low} ADAM17 ^{Low}	10	6		10	6		15	2	0	
CD44/ADAM17										
CD44 ^{High} ADAM17 ^{High}	8	14	0.9369	16	10	0.4762	9	12	8	0.0346*
CD44 ^{High} ADAM17 ^{Low}	6	4		6	6		10	3	1	
CD44 ^{Low} ADAM17 ^{High}	5	1		2	4		4	2	0	
CD44 ^{Low} ADAM17 ^{Low}	7	4		6	5		9	2	0	
Cases	26	23		30	25		32	19	9	

^aEleven cases of OSCC patients without age details.

^bFive cases of OSCC patients without gender details.

* $P \leq .05$; *** $P \leq .001$.

treatment of 0.5 μ M of CDDP. To confirm the sensitivity of FaDu-CDDP-R to CDDP exposure, we performed MTT assay to assess the drug sensitivity in terms of cell viability of parental and resistant cell line against CDDP treatment (1–5 μ M). As shown in Figure 2A, parental FaDu cells (FaDu-P) were found to be significantly more sensitive to CDDP than the resistant FaDu cells (FaDu-CDDP-R). Moreover, it is reported that mild therapeutic stress can induce stem-like, drug-tolerant stress-response states.¹³ To further investigate the effect of

CDDP exposure on acquisition of stemness in OSCC, the FaDu-P and FaDu-CDDP-R cells were plated onto ultralow attachment plates and sphere forming activity was observed and quantified after for 10 days. Notably, the resistant FaDu cells produced larger spheres than the parental FaDu cells (Figure 2B). We further noticed that FaDu-CDDP-R formed more ospheres (117 ± 11) when cultured in ultralow attachment plates, as compared with the parental FaDu P cells (44 ± 5) (Figure 2C). Furthermore, we analysed the expression of stemness

TABLE 3 Relationship between triple high expression and triple non-high expression of CD44/ABCB1/ADAM17 and the clinico-pathological features of OSCC

Groups	Age in years			Gender			Tumour grades			
	≤51	>51	P value	Male	Female	P value	Grade I	Grade II	Grade III	P value
CD44/ABCB1/ADAM17										
Triple high cases	22	19	1.000	21	21	0.3406	30	14	2	0.0001****
Triple non-high cases	4	4		9	4		2	5	7	
Cases	26	23		30	25		32	19	7	

Five cases of OSCC patients without gender details; 11 cases of OSCC patients without age details.

****P ≤ .0001.

marker and self-renewal marker β-catenin expression in both resistant and parental cell line through immunofluorescence microscopy and detected that FaDu-CDDP-R cells displayed higher β-catenin expression than the FaDu-P cell (Figure 2D). Figure 2E represents the corrected total cell fluorescence (CTCF) of β-catenin expression in parental FaDu cells and FaDu-CDDP-R cells showed significantly higher β-catenin expression in terms of fluorescence intensity as compared with FaDu-P cells. The higher CD44 expression as examined by flow cytometric analysis in FaDu-CDDP-R cells further confirmed the CDDP induced stemness in oral cancer (Figure 2F). In order to further advocate our hypothesis, we studied the changes in CD44⁺ population in FaDu-P and FaDu-CDDP-R group. CD44⁺ cells were reported to possess stem-like properties in various experimental setups. Excitingly, our flow cytometric analysis indicated an increase in percentage of CD44⁺ population in FaDu-CDDP-R group against FaDu-P which goes as 90 ± 2.6 and 36 ± 6.2, respectively. Similarly, the percentage of CD44⁻ population decreased from 64 ± 2.6 in FaDu-P group to 9 ± 2.6 in FaDu-CDDP-R group, suggesting therapy-induced stemness in OSCC (Figure 2G,H). To further correlate the expression of CD44, ABCB1 and ADAM17 with chemoresistance, we executed the immunofluorescence analysis of FaDu-P and FaDu-CDDP-R cells and discovered that FaDu-CDDP-R had higher level of expression of stemness marker CD44 (Figure 2I,J), drug-resistant marker ABCB1 (Figure 2K,L) and invasion mediator ADAM17 (Figure 2M,N) than the FaDu-P cells.

3.4 | CDDP-mediated autophagy regulates stemness of resistant cancer cells and expression of CD44, ABCB1 and ADAM17

Next, we investigated the autophagic response in FaDu-P and FaDu-CDDP-R cells. From the immunofluorescence analysis, we found augmented formation of punctate structure of LC3, a hallmark of autophagy induction in FaDu-CDDP-R cells with FaDu-P cells showed a diffused LC3 fluorescence with lesser puncta (Figure 3A). Approximately 24 ± 1.8% FaDu-P cells displayed LC3B-II puncta per cell, whereas it increased to 57 ± 5.9% in FaDu-CDDP-R cells (Figure 3B). Next, we evaluated the extent of autophagosome and autolysosome formation in terms of fluorescent LC3 puncta. Both the numbers of GFP and RFP puncta per cell were found in remarkable amount in FaDu-CDDP-R cells (Figure 3C). The number of

RFP⁺GFP⁺ (yellow) puncta was considerably higher in FaDu-CDDP-R cells indicating increased autophagosome formation (Figure 3C,D). Moreover, augmented autolysosome formation and higher autophagic flux in terms of the number of RFP⁺GFP⁻ (red) puncta per cell also significantly increased in FaDu-CDDP-R group as compared with their parental counterparts (Figure 3C,D). Further validating our hypothesis, the Western blot analysis also displayed increased conversion of LC3B-I to LC3B-II in FaDu-CDDP-R cells (Figure 3C,D). Moreover, authenticating the occurrence of increased autophagic flux in resistant cells, Western blot analysis showed noteworthy increase in the expression of autophagic signature molecules including ULK1, Beclin 1, ATG5, ATG7 and ATG14 in FaDu-CDDP-R cells as compared with FaDu-P cells (Figure 3E). In addition, enhanced degradation of p62 could be seen in FaDu-CDDP-R cells as compared with FaDu-P cells, confirming FaDu-CDDP-R cells exhibit greater autophagic flux (Figure 3E). To further investigate the role of autophagy in chemoresistance, we transiently down regulated autophagy induction by using siATG14 (Figure 3F) and studied the status of autophagy in siATG14 transfected cells and our data showed decreased formation LC3B-II puncta in ATG14 knocked down FaDu-CDDP-R cells as compared with FaDu-P cells. The percentage of cells positive for LC3B-II puncta decreased from 33 ± 2.8 to 18 ± 1.3 in parental FaDu cells and 78 ± 7.2 to 0.45 ± 4.1 when autophagy is inhibited (Figure 3G,H).

We further investigated mitophagic activity in CDDP-resistant FaDu cells through immunostaining of translocase of the outer mitochondrial membrane 20 (TOM20), an integral part of outer mitochondrial membrane. Our data showed that the percentage of cells with less/no mitochondrial TOM20 staining increased from 23 ± 2.2 to 60 ± 3.9 (Figure 4A,B) in FaDu-CDDP-R indicating the occurrence of mitophagy. In this connection, we analysed the expression of another mitochondrial marker COX-IV (cytochrome c oxidase subunit IV) which is an integral part of inner mitochondrial membrane in CDDP-resistant FaDu cells. From the Western blot analysis, it is clear that FaDu-CDDP-R cells have significantly lesser expression of COX-IV as compared with its parental counterpart (Figure 4C). To further substantiate our hypothesis that mitophagy plays a significant role in chemoresistance, we assayed functional mitophagy by using mitochondria-targeted mKeima-Red-Mito7, a lysosomal proteases resistant probe whose fluorescence shifts from green to red in acidic pH as an indicative of the mitochondrial delivery into

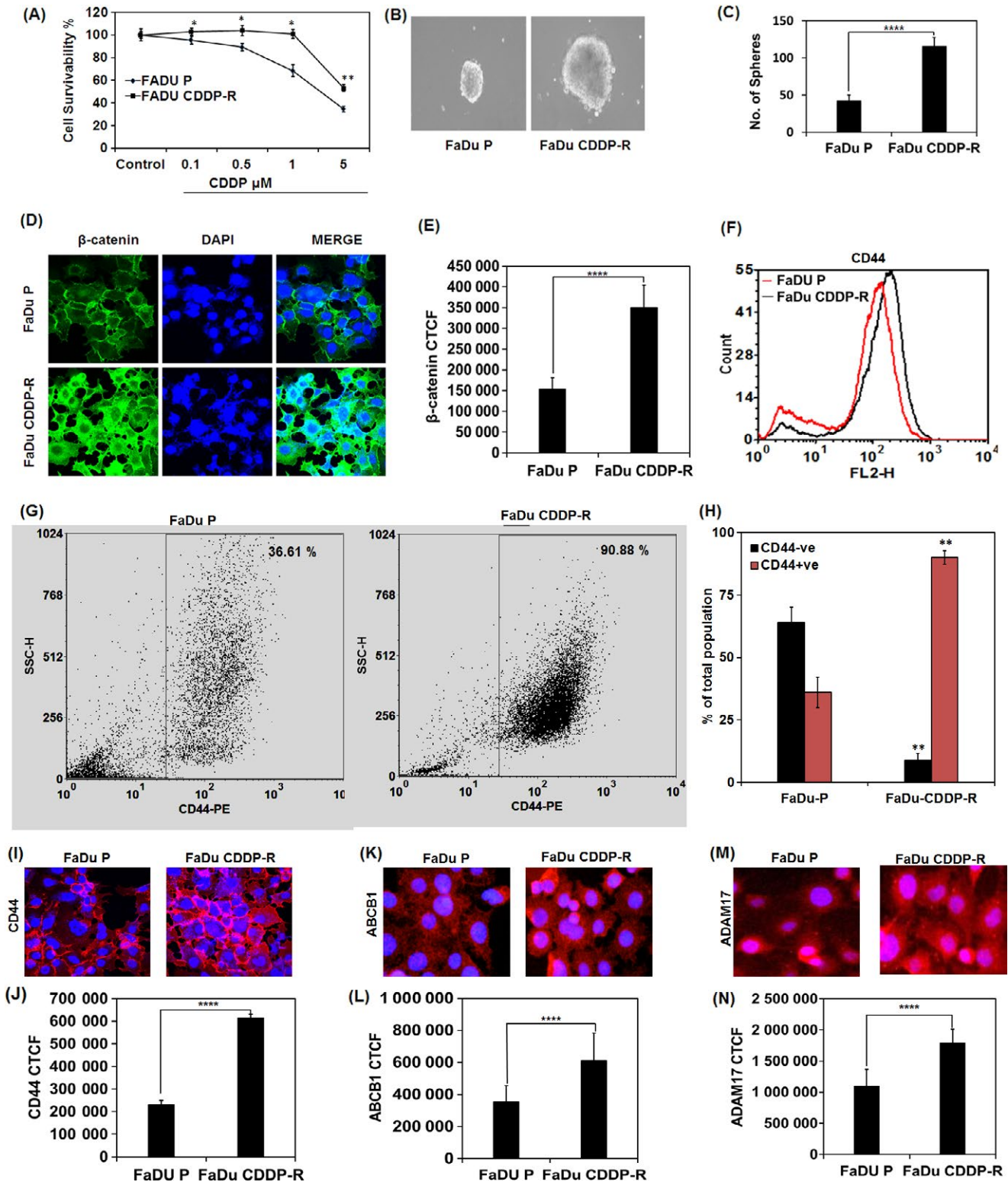


FIGURE 2 Cisplatin-resistant cells are endowed with cancer stem-like features and increased CD44, ABCB1 and ADAM17 expression. The FaDu-P and FaDu CDDP-R cells were treated with different concentration of CDDP for 72 h, and the cell viability was measured by MTT dye reduction assay (A). The FaDu P and FaDu CDDP-R cells were grown in ultralow attachment plate in stem cell media for 10 days, and the number and size of the orosphere produced were quantified (B, C). Expression of β -catenin in FaDu P and FaDu CDDP-R cells (D). The corrected total cell fluorescence (CTCF) of β -catenin expression as analysed by Image J software is depicted (E). Flow cytometric analysis of CD44 expression in FaDu-P and FaDu-CDDP-R cells (F). Flow cytometric analysis of CD44⁺ population with bar diagram represents the comparison between CD44⁺ and CD44⁻ population in FaDu-P and FaDu CDDP-R cells (G, I). Immunofluorescence image of indicated protein expression and their quantification in FaDu-P and FaDu CDDP-R cells (I-N). ***P* value ≤ 0.01 was considered significant when compared between FaDu-P and FaDu-CDDP-R

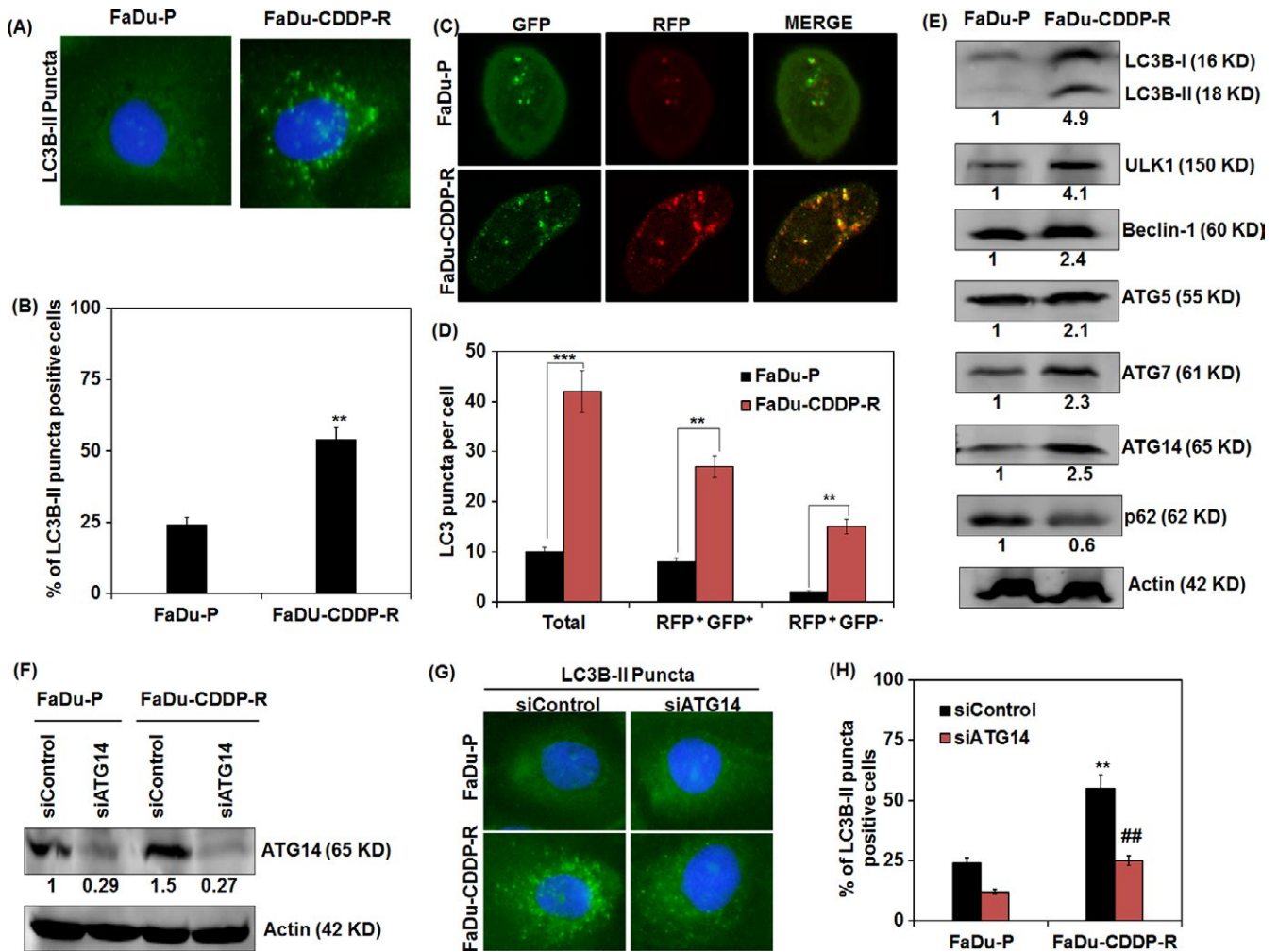


FIGURE 3 Role of autophagy in chemoresistance in FaDu cells. FaDu-P and FaDu-CDDP-R cells were analysed for the autophagy induction in terms of punctate fluorescence of LC3B-II expression (A) and quantification of % of LC3B-II puncta positive cells (B). Autophagic flux analysis in parental and resistant FaDu cells was done after tF-LC3 transfection, and the numbers of RFP*GFP* (yellow) and RFP*GFP- (red) puncta per cell were representing autophagosome and autolysosome, respectively, were counted (C and D). Western blot analysis of autophagic protein LC3B, ULK1, Beclin1, ATG5, ATG7, ATG14 and p62 is depicted (E). Western blot image depicts the down regulation of ATG14 with transient transfection of siATG14 in FaDu-P and FaDu-CDDP-R cells (F). Immunofluorescence analysis of LC3B-II puncta formation in FaDu-P and FaDu-CDDP-R cells in siControl and siATG14 transfected condition were evaluated (G). % of LC3B-II puncta positive cells in siControl and ATG14-deficient FaDu-P and FaDu-CDDP-R cells was quantified (H). Densitometry was performed on the original blots; the ratio of protein to actin in control cells was considered as 1. **P value < 0.01 was considered significant when compared between FaDu-P and FaDu-CDDP-R group. ##P value < 0.01 was considered significant when compared between FaDu-CDDP-R and siATG14- FaDu-CDDP-R

the maturing lysosome. As expected, a considerable increase in red mKeima-Red-Mito7 puncta per cell was found in FaDu-CDDP-R cells as compared with FaDu-P cells (Figure 4D,E). To further validate the induction of CDDP-mediated mitophagy in resistant cells, we investigated the expression of TOM20 in ATG14-deficient cells. The percentage of cells with less/no mitochondrial TOM20 in FaDu-P and FaDu-CDDP-R groups were 33 ± 2.8 and 78 ± 7.2 in siControl group. Intriguingly, the CDDP induced reduction in mitochondrial mass was reversed in siATG14 transfected group and the percentage of cells with less/no mitochondrial TOM20 in FaDu-P and FaDu-CDDP-R reduced to 18 ± 1.3 and 45 ± 4.1 (Figure 4F,G). Similar to the TOM20 expression, the immunoblotting showed efficient restoration COX-IV expression in ATG14-deficient FaDu-CDDP-R cells indicating the

potential association of mitophagy in chemoresistance of oral cancer (Figure 4H).

In order to justify our hypothesis, we inhibited autophagy and performed the spheroid formation assay. Interestingly, the numbers of orospheres produced were remarkably lower in ATG14-deficient FaDu-CDDP-R cells (86 ± 7.3) as compared to siControl group (266 ± 18.3) (Figure 5A). We then further analysed the self-renewal ability of capacity of FaDu-CDDP-R cells in terms of β -catenin expression. Our immunofluorescence data showed a considerable decrease in the β -catenin expression in ATG14-deficient-resistant FaDu cells against the siControl group, indicating the potential involvement of autophagy in maintaining self-renewal ability which is an indispensable characteristic of CSCs (Figure 5B,C). The inhibition of autophagy

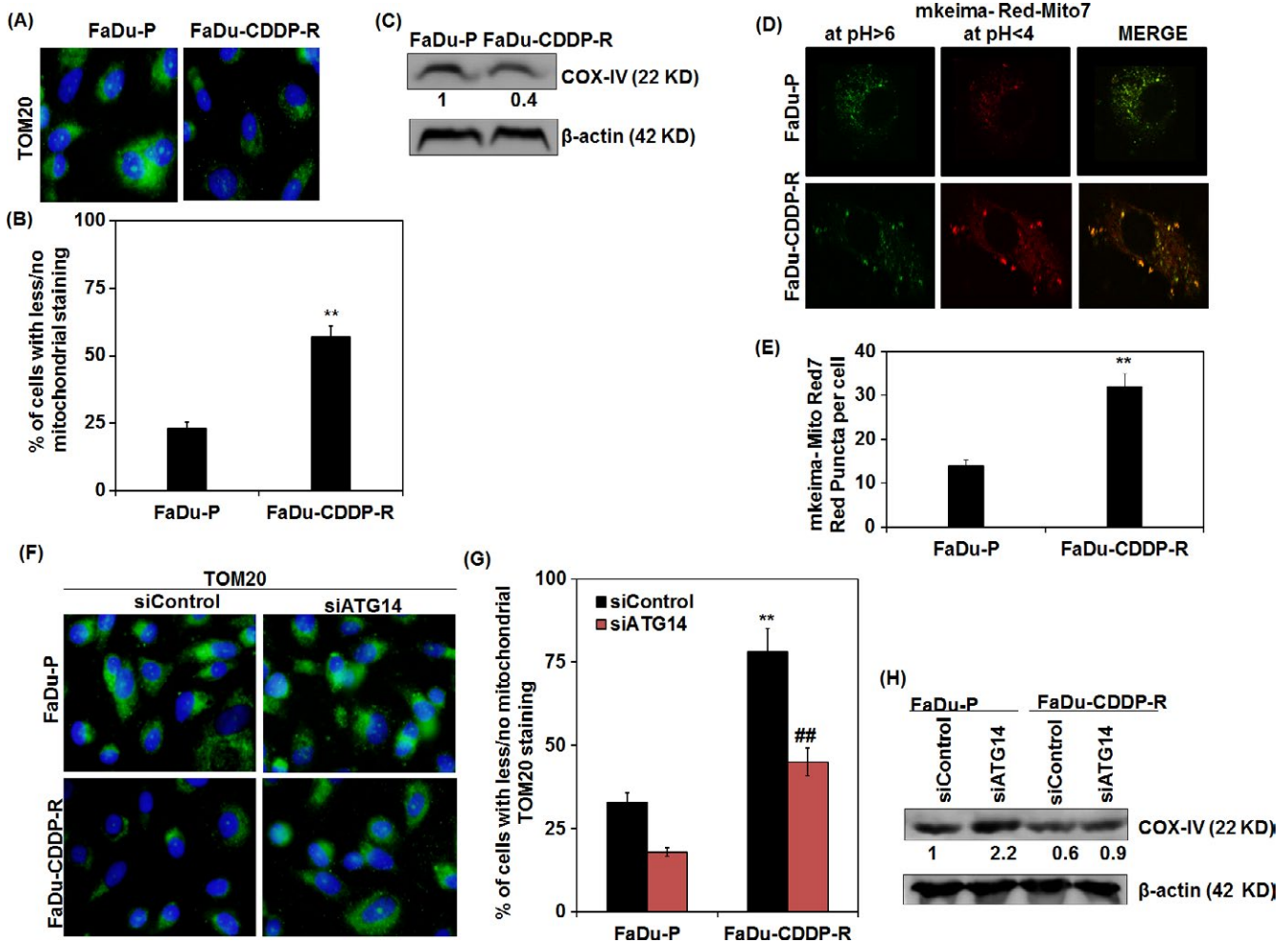


FIGURE 4 Mitophagy regulates chemoresistance in FaDu cells. Immunofluorescence expression (A) and quantification of outer mitochondrial marker protein TOM20 in FaDu-P and FaDu-CDDP-R (B) were analysed. Western blot analysis of inner mitochondrial marker protein COX-IV was investigated (C). The mitochondrial delivery to lysosome in order to measure the mitophagic flux (in terms of increased red puncta) was assayed by employing mKeima-Red-Mito7 (D and E). Immunofluorescence expression of TOM20 with quantification of % of cells with less/no mitochondrial TOM20 staining in FaDu-P and FaDu-CDDP-R cells after transient transfection of siControl and siATG14 are depicted (F and G). Western blot analysis of COX-IV expression in ATG14-deficient FaDu-P and FaDu-CDDP-R cells is represented (H). Densitometry was performed on the original blots, the ratio of protein to actin in control cells was considered as 1. ***P* value < 0.01 was considered significant when compared between FaDu-P and FaDu-CDDP-R groups. ##*P* value < 0.01 was considered significant when compared between FaDu-CDDP-R and siATG14- FaDu-CDDP-R

in FaDu-CDDP-R cells was found to effectively down regulate the stemness surface marker CD44 (Figure 5D,E) along with the down regulation of drug resistance marker ABCB1 (Figure 5F,G) and invasion mediator ADAM17 (Figure 5H,I), suggesting that autophagy regulates the expression of these proteins to induce stemness and chemoresistance. However, further study is required to understand how autophagy modulates the expression CD44, ABCB1 and ADAM17 during chemoresistance.

4 | DISCUSSION

Most of the cotemporary therapeutic regimens target only the bulk tumour mass leaving behind the assassin CSCs that serve as a reservoir for tumour repopulation post-therapy. Likely, CSCs in oral

cancer were reported to impose a great therapeutic implication in cancer relapse and patient survival.¹⁴ A correlation between CD44 expression and poor prognosis has been reported in many cancers.^{15,16} Moreover, CD44 may also symbolize a potential marker of OSCC stem cells.^{17,18} This study confirms the expression of stem cell surface marker CD44 expression in OSCC tissue samples. Moreover, CD44 expression in OSCC was found to be associated with tumour grade and tumour differentiation. Again, CSCs have been postulated to be drug resistance phenotypes by virtue of the expression of several drug efflux transporters.^{19,20} Previous study showed that expression of ABCB1 marker correlates with the degree of differentiation in gastric cancer.⁸ Similarly, in our study, the expression of ABCB1 highly correlated with the degree of differentiation of OSCC tissue. Moreover, the upregulated expression of ADAM17 was shown to be associated with poor prognosis of

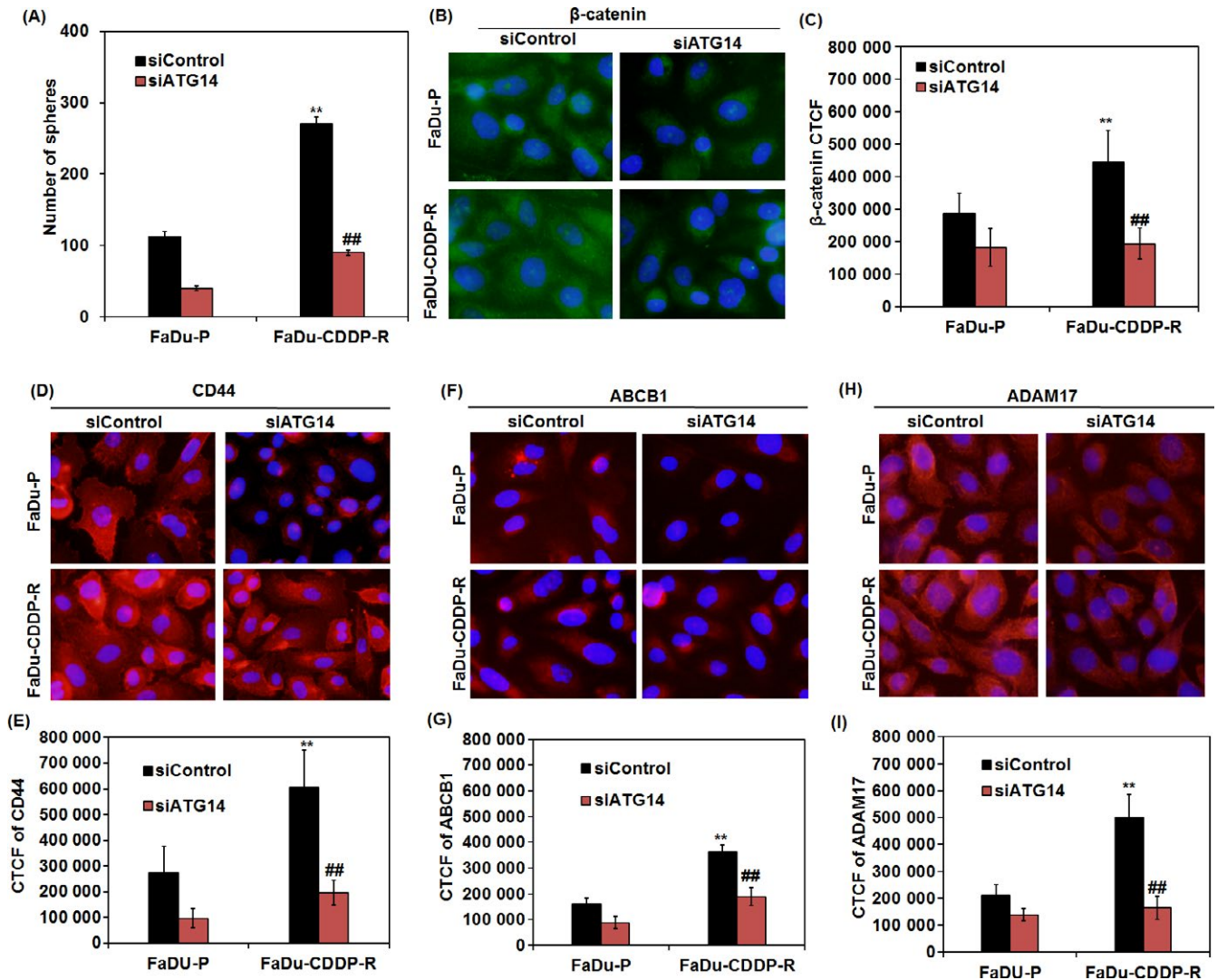


FIGURE 5 Autophagy regulates stemness and expression of CD44, ABCB1 and ADAM17 in CDDP-resistant FaDu cells. After knock down of ATG14, FaDu P and FaDu CDDP-R cells were grown in ultralow attachment plate in stem cell media for 10 days and the number of spheres produced were quantified (A). Immunofluorescence analysis of β-catenin, CD44, ABCB1 and ADAM17 in ATG14-deficient FaDu-P and FaDu-CDDP-R cells and their corresponding quantification was analysed (B-I). **P value < 0.01 was considered significant when compared between FaDu-P and FaDu-CDDP-R group. ##P value < 0.01 was considered significant when compared between FaDu-CDDP-R and siATG14- FaDu-CDDP-R

cancers.^{21,22} Likewise, we found that ADAM17 expression in OSCC significantly correlated with degree of cancer progression and aggressiveness.

While CD44 populations may indeed encompass a subpopulation of CSCs, by itself it does not appear to be an adequate stem cell marker for OSCC. Hence, the histological or clinical implications of CD44⁺/ABCB1⁺/ADAM17⁺ cells as putative CSCs in OSCC and their role in chemoresistance have been elucidated in this study. Higher tumour grades and poor tumour differentiation are one of the basic indicative features of cancer cell aggressiveness.^{23,24} A remarkable expression of CD44⁺/ABCB1⁺/ADAM17⁺ was obvious in higher OSCC tumour grade. Our data advocate that tumours with higher grades are more likely to encompass CSCs than the lower grade tumours. Likewise, marked CD44⁺/ABCB1⁺/ADAM17⁺ expression was evident in the component of OSCC showing poor

differentiation. This study also elucidates that morphologically, CSCs may show poorly differentiated (dedifferentiated) characteristics. This is consistent with a report by Ohara et al. indicating that poorly differentiated colon cancers contained more CSCs than well-differentiated tumours.²⁵ Therefore, our data imply that poor differentiation might be common characteristics of CSCs in clinical specimens.

Our study showed that the resistant FaDu cells had more stem characteristics as compared to parental cells as evident with the higher sphere forming capacity, CD44 and β-catenin expression. Accordingly, Nor et al. reported that stemness attributes of cancer cells is highly influenced by chemotherapeutic drugs and anticancer therapies and elucidated that cisplatin could induce the stem cell fraction in UM-SCC-22B HNSCC cells.²⁶ Moreover, the increased expression of CD44, ABCB1 and ADAM17 in resistant FaDu cells

emphasized the role of these proteins in chemoresistance. Likely, chemoresistance in colon carcinoma cells was reported to be mediated by CD44 survival signalling via Lyn Kinase and phosphoinositide 3-kinase/Akt.²⁷ In addition, it reported that CD44 expression was associated with chemoresistance in T-cell acute lymphoblastic leukaemia mediated in part through enhanced drug efflux system.²⁸ The chemoresistance in CSCs is mainly alleged to the higher expression of ABC drug efflux transporters.² The expression of ABCB1 in colorectal cancer has been shown to promote stemness and chemoresistance.²⁹ In consistent with the previous report,⁹ ADAM17 expression was also reported to be involved in stemness and chemoresistance in OSCC.

Many reports claim that autophagy in cancer cells promotes chemoresistance. Likely, our data show that resistant FaDu cells had higher autophagic flux as compared with parental counterpart which is in accordance with a report by Wang et al. which document that miRNA-25 regulates chemoresistance-associated autophagy in breast cancer cells.³⁰ Moreover, Song et al. also reported that hypoxia-induced autophagy is responsible for chemoresistance in hepatocellular carcinoma cells.³¹ Mitophagy is a selective form of autophagy where mitochondria are selectively degraded in autophagolysosomes. Though there are substantial reports on the involvement of mitophagy in cancer and many neurodegenerative diseases, there is no report on the association of mitophagy and chemoresistance.⁵ We, for the first time, showed that mitophagy is responsible for chemoresistance in oral cancer. However, the potential of specific targeting of mitophagy as opposed to bulk autophagy in general as a therapeutic strategy remains to be elucidated. Furthermore, previous studies have demonstrated that autophagy acts as a protective mechanism in CSCs in many cancers including osteosarcoma, urothelial carcinoma, colorectal cancer, bladder cancer, brain cancer and breast cancer offering a greater survival and chemoresistance advantage.^{6,32-36} Recently, it has been shown that JAK-mediated autophagy is responsible for maintenance of stemness in cisplatin-resistant bladder cancer cells.⁶ Moreover, in urothelial carcinoma cells, gemcitabine- and mitomycin-induced autophagy was also found to regulate cancer stem cell pool.³³ In accordance with previous studies and validating our hypothesis, the present investigation documents the autophagic inhibition in CDDP resistance in FaDu cells is associated with the down regulation of stem properties. Previous reports have demonstrated that osteopontin (OPN) triggers autophagy activation via the integrin/CD44 and p38 MAPK-mediated pathways.³⁷ Moreover, Whelan et al. reported the potential implication of mitophagy in regulation of tumour cells with high CD44 expression.³⁸ Likely, our study has demonstrated that inhibition of autophagy leads to the declined expression of CD44 in CDDP resistant FaDu cells indicating a potential link between CD44 expression and autophagy mediated chemoresistance. Interestingly, it was also reported that ABCB1 expression is positively correlated with LC3, Beclin1, Rictor expression and negatively correlated with the expression of Raptor in colorectal cancer patients, suggesting a strong association of autophagy in cancer progression

and multidrug resistance which is in agreement with this study involving the autophagic regulation of ABCB1-mediated drug resistance in oral cancer. However, paradoxically, it has been found that both autophagy inhibitor and inducer enhanced the activity of α secretases like ADAM10 and ADAM17.³⁹ Moreover, it has also been described that ADAM17 inhibitor, TIMP3 reduction causes a concomitant STAT1-dependent loss of FoxO1 activity, which consequentially diminishes protective autophagy genes to fuel glomeruli damage suggesting the prospective association of ADAM17 and autophagy process.⁴⁰ Accordingly, autophagy was shown to regulate the expression of ADAM17 in resistant FaDu cells which is a key molecule in the process of carcinogenesis and metastasis. As reported earlier, CDDP induces Mitochondrial-ROS Response that leads to mitochondrial dysfunction as a part of its cytotoxicity.⁴¹ Dysfunctional mitochondria are a hub of ROS generation which is encountered by selective clearance of reactive mitochondria through mitophagy.⁴² The basal level of mitophagy is higher in resistant cells as compared with the parental cells, suggesting mitophagy as a protective response in chemoresistant cells. Moreover, stem cells are reported to keep their basal redox status at a low level through inducing mitophagy,⁴³ which is in consistent with our investigation that higher mitophagy in resistant cell might be a driving force in the acquisition of stemness in oral cancer.

In conclusion, we have established that CD44⁺/ABCB1⁺/ADAM17⁺ cells have histologically poor differentiation, indicating that they are responsible for tumour aggressiveness. This simple eccentricity may manage CD44⁺/ABCB1⁺/ADAM17⁺ expression as a useful biomarker. We expect that our present results designating the clinical significance of CSC markers will contribute to new insight in targeting CSCs as well as address a number of problems associated with CSC biology. Further, we have established that selective mitochondrial autophagy (mitophagy) might be responsible for inducing chemoresistance in oral cancer. Moreover, our finding indicates that autophagy promotes stemness and drug tolerance in OSCC.

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CONFLICT OF INTEREST

The authors disclose no conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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