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ALDH1 & CD133 in invasive cervical carcinoma & their association with the outcome of chemoradiation therapy

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***Background & objectives*: Chemoradiation is the standard therapy for locally advanced invasive cervical cancer and response to treatment determines the outcome. Cancer stem cells (CSCs) and epithelial–mesenchymal transition (EMT) play a role in response to treatment and hence the aim of this study was to evaluate if their levels in pre-treatment biopsies by immunohistochemistry (IHC) could predict response to treatment and outcome.**

***Methods*: The study comprised 60 patients with FIGO Stage IIB/III invasive cervical carcinoma treated by chemoradiation. They were divided into two groups based on their clinical outcome: group 1, 30 patients who had no evidence of disease at 48 month follow up and group 2, 30 patients who had disease relapse within 6-12 months of treatment completion. IHC was performed for CSC markers (ALDH1, CD133, Nanog and Oct-4), EMT markers (E-cadherin and vimentin) and squamocolumnar junction (KRT7) markers and H-scores determined. Intergroup comparison was performed. The expression of these markers was also evaluated in histological sections of cervical pre-cancer (CIN1 and CIN3) in comparison to normal cervix.**

***Results*: Cervical Intraepithelial Neoplasia grade 3 (CIN3) showed high expression of ALDH1 and KRT7 as compared to normal cervical epithelium. Aldehyde dehydrogenase 1 (ALDH1) and CD133 were overexpressed in 70 and 24 per cent cervical carcinoma cases whereas E-cadherin showed reduced expression in invasive carcinoma as compared to normal controls. ALDH1 overexpression was significantly associated with disease relapse in invasive cervical carcinoma treated by chemoradiation ($P<0.01$).**

***Interpretation & conclusions*: Determination of ALDH1 levels in pre-treatment cervical biopsies of invasive cervical carcinoma may be useful for prediction of response to chemoradiation, with high levels predicting for a poor response.**

Key words ALDH1 - cancer stem cells - CD133 - cervical cancer - E-cadherin - epithelial–mesenchymal transition - keratin 7

Cervical cancer is the fourth most common cancer in women worldwide and accounted for 311,000 deaths in 2018[1]. The age-standardized incidence and mortality varies widely in different regions of the world. Shrestha *et al*[2], reviewed the scenario of cervical cancer in low- and middle-income countries and found that the highest

reported age-standardized mortality rate was 16/100,000/year in India in 2015. Concurrent chemoradiation with brachytherapy is the preferred modality of treatment for locally advanced cases[3,4]. The five-year overall survival for patients in FIGO (International Federation of Gynecology and Obstetrics) stage III/IV who reported to be 55.5 per cent in a Japanese cohort[5] whereas Shrivastava *et al*[6] reported 54 per cent survival in FIGO stage IIB treated by concurrent chemoradiotherapy. Further analysis of the patterns of failure revealed locoregional failure alone in 17.3 per cent, locoregional and distant failure in 10.8 per cent and distant only failures in 15.9 per cent[6]. In patients who present with local relapse, one possible reason could be intrinsic chemoradiation resistance. In this context, the role of cancer stem cells (CSCs) assumes importance as these are one of the causes of treatment failure in cancers. In a previous report, we observed an upregulation of CD133, a marker of CSCs in carcinoma cervix[7]. Similar observations have been made by others[8,9]. However, analysis of CSCs and their markers requires fresh samples which may not always be available, especially in cervical cancer, wherein the diagnosis is made by small biopsies. In this study, we explored the utility of immunohistochemical detection of CSC markers such as CD133 and the other related markers of 'stemness' such as aldehyde dehydrogenase 1 (ALDH1), Oct-4 and Nanog in patients with invasive cervical cancer following chemoradiation.

Epithelial–mesenchymal transition (EMT) is another important aspect linked to CSCs and may also play a role in determining the response to chemoradiation therapy[10-13]. The main markers of EMT are vimentin and E-cadherin, which were evaluated in conjunction with CSC markers. Finally, cells residing at the squamocolumnar junction (SCJ) have been implicated in cervical carcinogenesis and show expression of keratin 7 (KRT7) which have also been evaluated immunohistochemically[14]. The primary objective of the study was to evaluate the differences in expression of these markers in patients with either good or poor outcome following chemoradiation therapy in invasive cervical carcinoma. The secondary objective was to ascertain their expression in cervical pre-cancer and in normal cervix controls.

Material & Methods

This pilot study was conducted at the department of Cytology and Gynecological Pathology, Postgraduate Institute of Medical Education & Research, Chandigarh, India, after prior approval of the study protocol by the Institute Ethics Committee [vide letter no. NK/2795/Ph.D./1566].

Table I. Comparison of clinical and pathological characteristics of patients (n=60)

Characteristics	NED (n=30)	ED (n=30)
Mean age (yr) (range)	50.2 (33-75)	49.58 (25-70)
Tumour histology		
Squamous cell carcinoma	25	26
Adenocarcinoma	5	4
FIGO stage		
IB2	3	0
IIB	16	20
IIB	11	9
IV	0	1

NED, no evidence of disease; ED, evidence of disease; NS, not significant; FIGO, International Federation of Gynaecology and Obstetrics

A total of 60 consecutive patient with invasive cervical carcinoma, all in FIGO stage IIB/III who were treated by chemoradiation and brachytherapy during 2014-2015 with complete follow up details available, were included in the study. All patients were treated with the protocol consisting of administration of 46 Gy/23 # external beam radiation concurrently with 40 mg/m^2 cisplatin weekly dose with three-dimensional conformal radiotherapy using a four-field box technique followed by intracavitary brachytherapy (two fractions of 9 Gy high-dose rate delivered one week apart). All patients were followed up clinically with relevant clinical and radiological investigations performed which included haemogram, detailed clinical examination, biochemical evaluation including renal and liver function tests, ultrasonography and computed tomography (CT) scan of pelvis, abdomen and chest. All patients were followed up every two months for the first year, every three months until five years and thereafter six monthly after five years. Based on their clinical outcome the patients were grouped as good responders who showed no evidence of disease (NED) even after 48 month follow up and poor responders who showed evidence of disease (ED) in the form of local relapse within 6-12 months of follow up or who continued to have residual disease (Table I). In addition, 10 control samples [cervix with normal squamo-columnar junction (SCJ)] and 10 samples each of cervical intraepithelial neoplasia grade 1(CIN1/low-grade squamous intraepithelial lesion) and CIN2/3

(high-grade squamous intraepithelial lesion) were also evaluated to determine their role if any, in cervical precancerous lesions.

Immunohistochemistry (IHC) was done using an automated immunostainer Benchmark XT Ventana machine (Roche, USA). Sections (3-4 µm) from each tissue biopsy block were placed onto a poly-L-lysine-coated slide and the manufacturer's protocol was followed. The primary antibodies used were against ALDH1 (clone L1, Abcam, USA, 1:50 dilution), Nanog (clone D73G4, Cell Signalling, USA, 1:200), CD133 (cloneD4W4N, Cell Signalling, USA, 1:50), Oct-4 (clone MRQ-10, Cell Marque, USA, 1:200), vimentin (V9 clone, Cell Marque, USA, ready to use), KRT7 (clone Ov-TL 12/30, Dako, Denmark, 1;200) and E-cadherin (clone EP700Y, 1:100). The IHC-stained slides were visualized and scored by light microscopy. The positive control section included a case of breast invasive ductal carcinoma for ALDH1 and E-cadherin, normal kidney section for CD133, dysgerminoma section for Oct-4 and Nanog and ovarian serous carcinoma for KRT7. The negative control in each case was served by omission of primary antibody.

Scoring of immunostaining: Scoring of IHC was done independently by two observers (RS and SB) by light microscopic evaluation using an Olympus BX50 microscope (Olympus, Japan). A previously described scoring system taking into account percentage positivity and staining intensity was applied[15]. An intensity score of 0 (no staining), or 1 (weak), 2 (moderate) and 3 (strong) was given. The IHC score was recorded by multiplying the percentage positivity with intensity score obtained with final H-score ranging from 0 to 300.

Statistical analysis: The mean and median values of H-scores were calculated. The statistical software GraphPad Prism 7.0 for windows (InStata, GraphPad Software Inc. CA, USA) was used to derive dot plots and apply Student's t test on H-scores of the NED and ED groups.

Results

Aldehyde dehydrogenase (ALDH): ALDH1 immunohistochemical expression was predominantly cytoplasmic (Fig. 1). Mild diffuse positivity was seen in the basal layer of the normal squamous epithelium and endocervical glands (Fig. 1A); increased expression was seen in squamous metaplasia (Fig. 1B). Cervical intraepithelial neoplasia grade 1 (CIN1) showed nil-to-mild expression of ALDH1 (Fig. 1C) whereas CIN3 showed high expression (Fig. 1D). Of the 60 patients with invasive cervical carcinoma cases 42 (70%) showed ALDH1 expression ranging from 1+ to 3+ (Fig. 1E-H). The basal layer or tumour cells along the epithelial–stromal interface showed moderate levels (Fig. 1F). Focally increased expression was observed in tumour cells in necrotic foci and around blood vessels, especially in the relapsed cases.

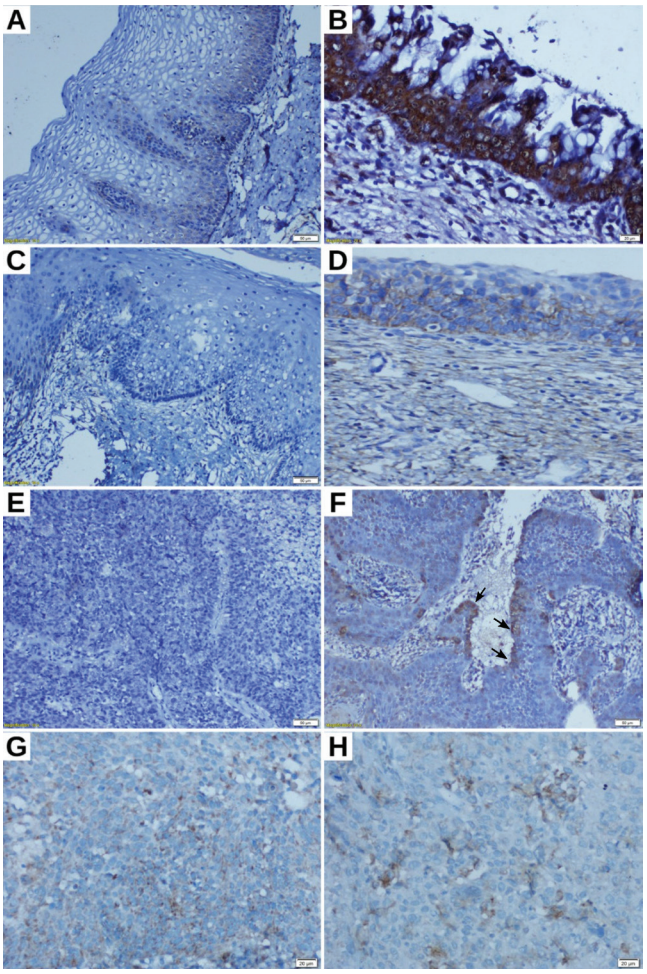

Fig. 1. Aldehyde dehydrogenase 1 immunohistochemistry panel. (**A**) Normal squamous epithelium showing weak basal positivity; (**B**) squamous metaplasia with strong diffuse positivity; (**C**) CIN1, no expression; (**D**) CIN3, showing moderate positivity; (**E-H**) squamous cell carcinoma with no expression (**E**), weak expression at stromal interface (black arrows) (**F**), scattered moderate expression **(G)** and patchy strong expression (**H**) (×200, Immunoperoxidase stain).

CD133: CD133 protein expression was found to be cytoplasmic and focally membranous. The positive control was a section of the kidney showing expression in the proximal convoluted tubules (2G). Endocervical surface epithelium and glandular

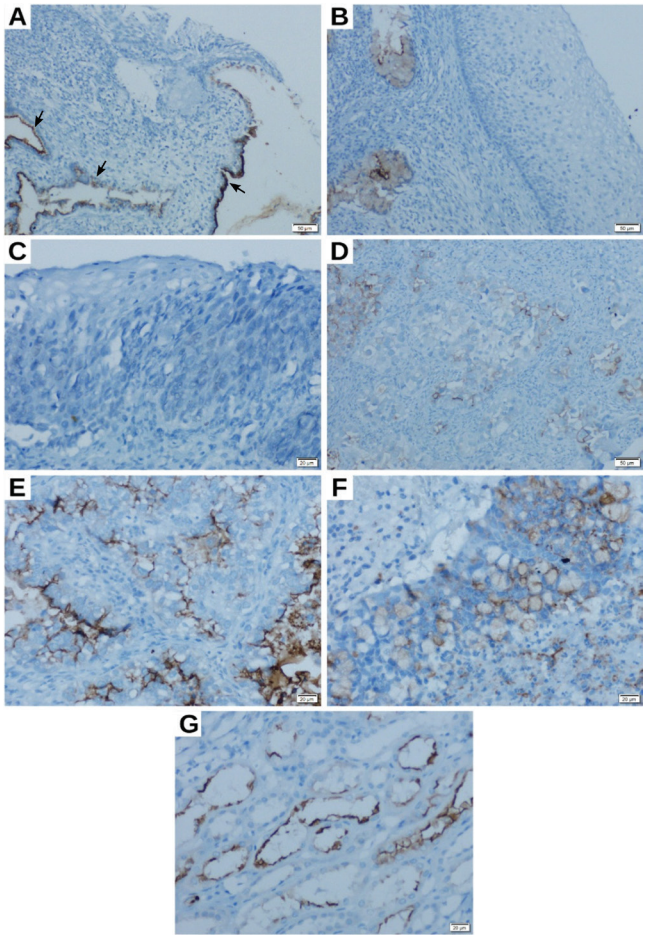

Fig. 2. CD133 immunohistochemistry panel. (**A**) Squamocolumnar junction showing expression in endocervical epithelium and glands (black arrows) and negative squamous epithelium; (**B**) CIN1 and (**C**) CIN3, negative expression. (**D**) Squamous cell carcinoma **(E)** adenocarcinoma showing heterogeneous expression, respectively, and (**F**) upregulated expression in tumour surrounding necrotic focus and (**G**) normal kidney section (positive control) (×200, Immunoperoxidase stain).

lining epithelium showed luminal and membranous positivity; however, the squamous epithelium was negative for CD133 (Fig. 2A and B). At the SCJ, only the endocervical epithelium was highlighted (Fig. 2B). All cases of CIN1 and CIN3 were found to be negative for CD133 expression. Invasive cervical cancer cases showed CD133 expression in 23 per cent (14/60) (Fig. 2C-F). Expression was patchy and heterogeneous, seen as tiny islands of 15-20 positive cells, close to the stroma. Focally increased expression was observed in tumour cells in necrotic foci (Fig. 2F).

Nanog: Nanog expression was predominantly cytoplasmic. Normal cervix including the SCJ did not show Nanog expression. Of the 10 cases of

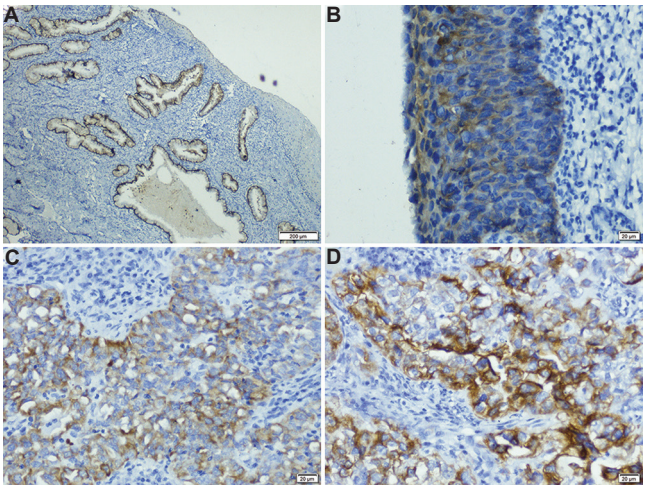

Fig. 3. KRT7 immunohistochemistry panel. (**A**) Squamocolumnar junction showing no expression in the squamous epithelium and positivity in the endocervical surface and glandular epithelium. (**B**) CIN3 showing high levels; (**C** and **D**) squamous cell carcinoma showing heterogeneous expression (×20, Immunoperoxidase stain).

CIN1 evaluated, three showed focal positivity. All CIN3 cases were negative for Nanog expression. Invasive cervical cancer cases (n=60) showed patchy heterogeneous expression in 23 per cent cases (14/60).

Oct-4: Oct-4 was not expressed in any of the cases including normal controls, CIN and invasive cancer although the antibody stained the positive control section of a dysgerminoma.

Keratin 7: In the SCJ and normal cervix, cytoplasmic KRT7 positivity was in the endocervical cells and glands and negative in the squamous cells (Fig. 3A). CIN1 was negative or showed weak and focal positivity. CIN3 showed positivity ranging from 2+ to 3+ (Fig. 3B). Invasive cervical cancer cases (n=60) showed KRT7 expression in 66.6 per cent cases (40/60) with variable staining intensity (Fig. 3C and D).

E-cadherin: The normal cervix showed diffuse cytoplasmic and membranous positivity ranging from 2+ to 3+ in the squamous epithelium which was maximum in the lower third and showed decreasing intensity with maturation of the epithelium (Fig. 4). The endocervical surface lining as well as the glandular lining also showed similar strong positivity. Squamous metaplasia, CIN1 and CIN3 all showed diffuse strong positivity for E-cadherin. Invasive cervical cancer cases showed E-cadherin expression in 96.6 per cent (58/60) cases. Further, most tumours showed intratumoural heterogeneity with respect to E-cadherin expression with focal

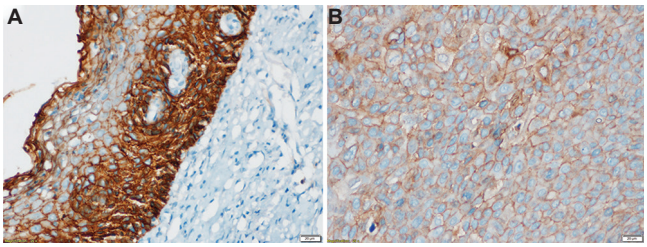

Fig. 4. E-cadherin immunohistochemistry panel. (**A**) Normal squamous epithelium showing diffuse strong expression and; (**B**) Invasive squamous cell carcinoma showing reduced levels of expression (×20, Immunoperoxidase stain).

strong positivity and large areas with weak expression. Tumour around the areas of necrosis showed higher levels.

Vimentin: Vimentin expression was predominantly cytoplasmic. All normal cervix controls and cases of CIN1 and CIN3 were negative. In invasive cancer, occasional positive cells amounting to <5 per cent of tumour area were noted. In only one case, there was a high vimentin expression with an H-score of 200. Overall, H-scores could not be computed as levels of expression were low.

Prediction of response to chemoradiation therapy: There were 30 patients with NED and 30 with local failures or ED. The two groups did not differ with respect to mean age, histological type of carcinoma (squamous cell carcinoma), FIGO stage, tumour grade and treatment modality. Table II shows the difference in H-scores of the markers evaluated. Only ALDH1 showed a significant difference between the two groups (P<0.01) being upregulated in the ED (relapsed cases) group as compared to the NED group (Table II). No specific cut-off score was derived. The combined analysis of ALDH1 and CD133 H-scores was also done and the results were non-significant.

Discussion

Chemoradiation is the mainstay of treatment of locally advanced invasive cervical carcinoma and intrinsic resistance to radiation therapy is the most important cause of treatment failure. It was hypothesized that levels of expression of CSC, stemness and EMT markers could be involved in determining the outcome to chemoradiation. In this pilot study, pre-treatment cervical biopsies from patients with invasive cervical carcinoma with a good outcome were compared with those with a poor outcome for the above markers.

Members of the aldehyde dehydrogenase (ALDH) family of proteins, especially ALDH1, have been implicated in radiation resistance and tumour recurrence in several cancers. ALDH1 is a general functional marker of CSCs in tumours[16]. The antibody against ALDH used was against the major isoform ALDH1 with immunoreactivity present in the basal cells of the squamous and glandular epithelium as reported previously in prostate[17]. These findings are expected as the basal cells are important for continuous epithelial turnover and hence require ALDH1, a molecule implicated in stemness. The marked upregulation of ALDH1 in squamous metaplasia was a novel observation. Further, high expression in CIN3 but not in CIN1 warrants further investigation into this potential marker for high-grade dysplasia. Overall 70 per cent cases showed ALDH1 expression which was higher as compared to 24 per cent reported previously[18]. Our observations were consistent with a report of ALDH1A1 upregulation in cervical cancer tissues which was also reflected in the circulating plasma[19]. Although no specific niche for ALDH1 positivity was noted, areas surrounding necrotic foci and the epithelial–stromal interface showed more positive cells indicating their relationship with hypoxia and invasion. Tissue necrosis is the result of hypoxia leading to nutrient and oxygen deprivation and the necrotic content of tumour cells enhances angiogenesis, migration and invasion[20].

CD133 is a phenotypic marker of CSCs identified by our group[7] and by others[8,9,21,22]. CD133 was expressed in the endocervical lining epithelium but not in the squamous epithelial lining of normal cervix. CD133 had little or no role in cervical pre-cancer progression as all cases of CIN were negative. Cervical carcinoma showed expression in only 23 per cent cases which was heterogeneous with luminal membranous positivity in adenocarcinoma and increased expression around necrotic foci of tumour cells in a few squamous cell carcinomas. Using frozen tissues and immunofluorescence, Hermann *et al*[23] observed CD133+ CSC presenting at the invading front of pancreatic cancer and proposed them to be responsible for the metastatic phenotype. Lingala *et al*[24] showed co-localization of CD133 and ALDH1 in five per cent hepatocellular carcinoma cells by confocal within vessels and in connective tissue suggesting metastatic ability.

Nanog positivity was only observed in invasive cancer with no significant difference in the NED versus ED groups. Stromal cells showing positivity for Nanog have been shown to be associated with cervical

Table II. Comparison of H-scores of markers evaluated in no evidence of disease (NED) versus evidence of disease (ED) groups in invasive cervical carcinoma

Cancer stem cell makers	Positive (%) (n=60)	H-scores			
		NED (n=30)		ED (n=30)	
		Median	Mean±SD	Median	Mean±SD
ALDH1	42 (70)	10	36±66.2	40	55.8±59.8**
CD133	14 (23)	0	11.7±25.4	0	12±35.4
Nanog	14 (23)	0	8.6±21.6	0	7.1±16.3
E-cadherin	58 (96.6)	180	173.4±73.5	200	173.3±83.5
Keratin 7	40 (66.6)	50	69±73.7	60	72.6±76.6

**P<0.01 compared to NED group, ALDH1, aldehyde dehydrogenase 1

cancer progression[25]. Immunostaining for Oct-4 was unrewarding as it was negative in all cases evaluated in spite of repeated attempts and with good positive control sections. Yang *et al*[26] showed significantly high expression of Oct-4 in cervical cancer. In another study, Oct-4 expression was seen in 57 per cent cases and along with downregulation of SOX2 correlated with poor prognosis[27]. The contradictory results of our study could be due to the different clones of antibody used which were different from the study by Yang *et al*[26].

The SCJ cells are believed to play an important part in the progression of cervical cancer and Herfs *et al*[14] proposed that keratin 7 besides other markers identifies a unique population of cells at the SCJ which are important in cervical carcinogenesis. In our study, the SCJ, endocervical cells and glands showed expression for KRT7, and strong positivity was observed in CIN3 cases showing abrupt increase near the SCJ area. KRT7 showed a differential expression in CIN3 versus CIN1 and hence could serve as a useful biomarker for detection of high-grade CIN[28,29]. In invasive cancer cases, KRT7 expression was variable and seen in 66.6 per cent cases; however, there was no relationship with chemoradiation response and patient outcome. Lee *et al*[30] have suggested that CK7 (KRT7) expression may be related to viral episomal replication and in conjunction with CK19 may be a valuable marker of HR-HPV and E7 oncoprotein level in the tumour.

Decreased expression of E-cadherin in tumours as compared to diffuse high expression in normal cervix and variable expression in cervical pre-cancer have been reported[31-33] similar to our study. However, E-cadherin expression was not related to the outcome or the prognosis as previously reported[33]. Vimentin expression was low in cervical cancer cells. Gilles *et al*[34] correlated vimentin expression in invasive cervical carcinomas with lymph node metastases. Lin *et al*[13] reported vimentin positivity in 27 per cent cases which correlated significantly with age, lymphatic invasion, disease recurrence and nodal metastases indicating it to be a prognostic marker in patients who have undergone primary surgery. E-cadherin and vimentin are markers of EMT and the reduction in E-cadherin is the first step in EMT followed by upregulation of vimentin. While all tumours showed reduced E-cadherin expression indicating activation of the first step in EMT, vimentin upregulation was generally not observed.

The present analysis was done to evaluate the relationship of the important stemness and EMT markers with the chemoradiation response. Among all the markers tested, only ALDH1 expression showed a significant difference between the ED and NED groups with upregulation in the ED group. Hence ALDH1 overexpression in pre-treatment biopsies portends a poor outcome. All other stemness markers such as KRT7, CD133 and Nanog and the EMT markers of E-cadherin and vimentin failed to show any difference in the expression levels between the two groups. Our findings need to be validated in a prospective multi-institutional larger cohort of patients. Muralikrishnan *et al*[35] have reviewed inhibitors of ALDH which may be used to target CSCs in gynaecological malignancies for improving their outcomes. Our study demonstrated the feasibility of evaluation of ALDH1 levels in pre-treatment biopsies by IHC, which may be used for identification and recruitment of such patients into clinical trials to use novel therapies targeting ALDH1 that may improve their response to chemoradiation.

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***Conflicts of Interest*:** None.

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