Clinical significance of *Aldh1a1, Cd133* and *Oct 4* in breast cancer and its association with epithelial mesenchymal transition

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ABSTRACT

Objective: To evaluate role of stem cell markers *Aldh1a1, Cd133* and *Oct 4* and its association with Epithelial Mesenchymal Transition (EMT) markers in breast cancer patients.

Materials and method: Total of 100 breast cancer patients were enrolled. These markers were studied by immunohistochemistry method and correlated with clinicopathological parameters, ER, PR, *HER2* and disease status.

Results: *Aldh1a1* expression was noted in 43% patients with breast cancer and higher incidence was found in patients with age >45 years (p=0.02), postmenopausal status, *IDC*+ mucinous carcinoma and papillary carcinoma histological type and ER positivity. *Cd133* expression was noted in 87% patients with breast cancer and showed significant positive correlation with *Oct 4* expression (p=0.002) and vimentin (p=0.003). Univariate survival analysis showed an association of *Cd133* negative expression with reduced DFS and OS. *Oct 4* expression was noted in 84% patients with breast cancer and associated with poor OS. In multivariate survival analysis, for DFS lymph node entered at step 1 and *Cd133* negative expression entered at step 2. For OS, lymph node entered at step 1, *Cd133* negative expression entered at step 2 and *Oct 4* positive expression entered at step 3.

Conclusion: *Cd133* and *Oct 4* emerged as independent biomarkers to predict worse prognosis in breast cancer patients. *Cd133* may have a role in EMT and could be used as a drug target.

Key Words: *Aldh1a1, Cd133, Oct 4, Breast cancer.*

Abbreviations: DFS: Disease Free Survival; OS: Overall Survival

Cancer metastasis, resistance to therapies and disease recurrence are significant hurdles to successful treatment of breast carcinoma. Human breast cancers have been reported to contain a subpopulation of cancer cells similar to epithelial stem cells [1-3]. They have the ability to self-renew and undergo differentiation to phenotypically diverse populations of tumour cells [2]. Substantial evidence gathered over the last decade suggests that breast cancer progression and recurrence is supported by Cancer Stem Cells (CSCs) and understanding how CSCs form and contribute to the pathology of breast cancer will greatly aid the pursuit of novel therapies targeted at eliminating these cells. Stem cell markers such as *Aldh1a1*, *Cd133* and *Oct 4* are used to identify breast cancer stem cells. In mouse xenografts, *Aldh1a1*positive breast cancer cells are able to promote tumor invasion in vitro and promote tumor metastasis [4]. *Aldh1a1* expression was an independent predictive factor for early metastasis and decreased survival in inflammatory breast carcinoma. Also, high expression of *Aldh1a1 mRNA* is also observed to be correlated with poorer overall survival in breast carcinoma patients, and as a result, *Aldh1a1* is the only *Aldh1* isozyme capable of serving as a biomarker for predicting poorer survival in breast carcinoma patients [5]. *Cd133* is recognized as an important biomarker to identify and isolate the specific cell subpopulation named "cancer cells with stem cell phenotype" in many types of neoplasms including breast carcinoma. High *Cd133* expression cells shows higher invasive capability and increased expression of protein involved in metastasis and drug resistance of breast tumor [6,7]. *Oct 4* is well known for its key role in maintenance of self-renewal and pluripotency. It is also regarded as a marker of stem like cells in cancer. *Oct 4* is expressed in subpopulation of breast and ovarian cancer cells possessing self-renewal ability [8,9]. The presence of these markers is often associated with chemotherapy and radiotherapy resistance. Therefore, this study determined breast cancer stem cells by immunohistochemical localisation of *Aldh1a1*, *Cd133* and *Oct 4* markers and its association with clinicopathological parameters, ER, PR, *HER2* and disease status to identify marker of disease aggressiveness.

MATERIALS AND METHODS

Patients

This retrospective study was approved by institutional scientific and ethics committees, included 100 breast cancer patients diagnosed and treated at the Gujarat Cancer & Research Institute (Stage II A + II B, N=67 and Stage III A + III B, N=33). Detailed clinical history such as age, menopausal status, tumor size, lymphnode involvement, stage of disease, histopathological type, Bloom Richardson (BR) score, radiological findings, and treatment offered were recorded from the case files maintained at the Medical Record Department of the institute. Disease staging was done according to UICC TNM classification. Disease status was assessed by clinical examination, radiological investigations and biochemical investigations.

Immunohistochemical Localization

The tumor tissue blocks were obtained from the archives of Pathology Department of the Institute. 4 µm thin sections were cut on microtome (Leica, Germany) and taken on *3-Aminopropyl triethoxysilane* (APES) coated slides. Immunohistochemical localization of markers *Aldh1a1*, *Cd133* and *Oct 4* was performed on Formalin Fixed Paraffin Embedded (FFPE) tissue blocks containing primary tumor and evaluated by Haematoxylin and Eosin (H&E) staining, on Ventana Benchmark XT autoimmunostainer using Ventana reagents (Ventana, USA). Briefly the protocol includes following steps of deparaffinization using EZ solution, antigen retrieval for 60 minutes using retrieval solution Cell conditioning1 (CC1), and incubation with Ultra View DAB inhibitor for 4 minutes, 100 µl of respective primary antibodies of *Aldh1a1* (D9Q8E, 1:100, Cell signaling) for 32 minutes, *Cd133* (DF10, 1:100, GeneTex) for 32 minutes, *Oct 4* (*EP143*, 1:50, BioGenex) for 32 minutes, Ultra View HRP Multimer for 8 minutes, Ultra View DAB Detection kit for 8 minutes, counterstained with haematoxylin for 8 minutes, bluing reagent for 4 minutes and mounted with DPX.

Scoring

Two individual observers scored the sections. Cytoplasmic staining pattern was observed for *Aldh1a1*, membranous staining for *Cd133*, and nuclear staining for *Oct 4*. Histoscore (H-score) was evaluated by multiplying percentage of positive cells with the staining intensity for *Aldh1a1*, *Cd133* and *Oct 4*. H-score from 0 to 300 were evaluated where 0-50 was scored as negative (0), 51-100 as weak positive (1+), 101-200 as moderate positive (2+),

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and 201-300 as strong positive (3+). For statistical analysis, H-score 0 was scored negative, and 1+, 2+ and 3+ were clubbed as positive.

Statistical Analysis

Statistical analysis was carried out using SPSS statistical software version 20 (SPSS Inc, USA). Mean, Standard error (SE) of mean and median were calculated and Pearson's Chi-square test with Pearson's correlation coefficient (r) was used to assess correlation and significance between two parameters. In case of patient number less than 5 in the cells of 2x2 tables, Yates' Continuity correction value along with its significance was taken into consideration. Univariate survival analysis was carried out by Kaplan Meier and Log Rank statistics was used to assess the prognostic significance of Disease Free Survival (DFS) and Overall Survival (OS). Multivariate survival analysis was performed using Cox regression model with forward stepwise (likelihood ratio) method. The Wald statistics and relative risk with 95% Confidence Interval (CI) for were used to evaluate the prognostic significance. P values ≤ 0.05 were considered to be significant.

RESULTS

Patient's Characteristics and Outcome

This retrospective study included 100 patients, 38% patients had age ≤ 45 years, whereas 62% patients had >45 years. More than 50% of patients had postmenopausal status, T2 size tumors, lymph node negativity, stage II disease, histology grade II and III tumors, Invasive Ductal Carcinoma (IDC) and triple negative tumors (ER, PR and *Her-2-neu* negative). The primary treatment offered to all patients was surgery and adjuvant treatment with polychemotherapy [CMF (*cyclophosphamide+methoterxate+5-fluorouracil* n=6); CMF+TMX (*Tamoxifen* n=2); CMF+TMX+RT n=6; FAC (*5-fluorouracil +Adriamycin+cyclophoshamide* n=14); FAC+TMX n=14; FAC+RT+TMX n=55; Radiotherapy (RT) n=3]. For statistical analysis CMF alone and CMF with adjuvant therapy (CMF+TMX; CMF+TMX+RT) and FAC alone and FAC with adjuvant treatment (FAC+TMX; FAC+RT+TMX) were clubbed together. The maximum follow-up period was 96 months with a median follow-up was 62 months and 47% patients developed metastasis or local recurrence and 20% died due to cancer within study period. Patient's clinical and pathological characteristics are mentioned in Table 1.

***Aldh1a1* Expression:** Cytoplasmic *Aldh1a1* expression was observed in 43% of the tumors with H-score of 1+ in 13%, 2+ in 20% and 3+ in 10% in patients with breast cancer (Figure 1). A significant positive correlation of *Aldh1a1* expression was observed in patients with age >45 years (p=0.02). A trend of higher incidence of *Aldh1a1* expression was observed in patients with postmenopausal status, Invasive Ductal Carcinoma (IDC) + mucinous carcinoma and papillary carcinoma and ER positive status. No significant correlation was observed with other clinical and pathological parameters (Table1).

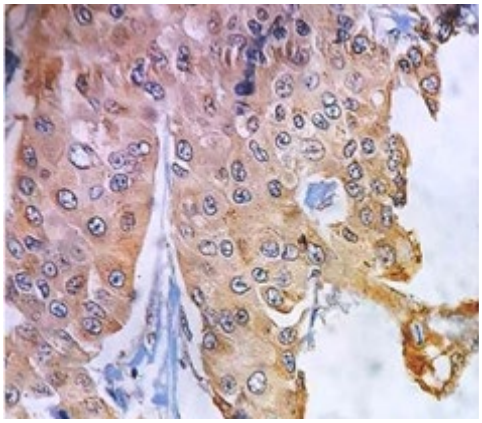

Figure 1) Cytoplasmic *Aldh1a1* depicted by brown staining in tumor cells of breast cancer.

***Aldh1a1* Expression in Relation to Survival:** Regarding Kaplan Meier univariate survival analysis with respect to DFS, similar incidence of disease relapse and mean months DFS was noted in patients with negative *Aldh1a1* expression (46%, 26/57; 60.41 ± 4.14 months) and positive *Aldh1a1* expression (49%, 21/43; 53.97 ± 4.30 months, Log rank= 0.19, df=1, p=0.65). With respect to OS, similar incidence of death and mean months of OS was noted in patients with negative *Aldh1a1* expression (19%, 11/57; 79.73 ± 2.88 months) and positive *Aldh1a1* expression (21%, 09/43; 71.34 ± 3.31 months, Log rank=0.17, df=1, p=0.67; (Table 2).

***Aldh1a1* expression in relation to treatment:** In relation to treatment, *Aldh1a1* expression was not found as an independent predictor of treatment response in relation to DFS and OS (Table 3).

***Cd133* Expression**

Membranous *Cd133* expression was observed in 87% of the tumors with H-score of 1+ in 04%, 2+ in 33% and 3+ in 50% (Figure 2). *Cd133* protein expression when correlated with clinical and pathological parameters, no

TABLE 1

Correlation of *Aldh1a1, Cd133* and *Oct 4* with clinical and pathological parameters

Parameters		*Aldh1a1* Expression		*Cd133* Expression		*Oct 4* Expression	
	N (%)	Negative N (%)	Positive N (%)	Negative N (%)	Positive N (%)	Negative N (%)	Positive N (%)
Age (Years)	100	57 (57)	43 (43)a	13 (13)	87 (87)	16 (16)	84 (84)
≤ 45 years	38 (38)	27 (71)	11 (29)	06 (16)	32 (84)	05 (13)	33 (87)
> 45 years	62 (62)	30 (48)	32 (52)	07 (11)	55 (89)	11 (18)	51 (82)
Menopausal Status	100	57(57)	43 (43)	13 (13)	87 (87)	16 (16)	84 (84)
Premenopausal	35 (35)	24 (69)	11 (31)	05 (14)	30 (86)	03 (09)	32 (91)
Postmenopausal	65 (65)	33 (51)	32 (49)b	08 (12)	57 (88)	13 (20)	52 (80)
Tumor size	100	57 (57)	43 (43)	13 (13)	87 (87)	16 (16)	84 (84)
T1 (≤ 2 cm)	06 (06)	04 (67)	02 (33)	00 (00)	06 (100)	00 (00)	06 (100)
T2 (≥ 2 cm-≤ 5 cm)	70 (70)	39 (56)	31 (44)	12 (17)	58 (83)	13 (19)	57 (81)
T3 (≥ 5 cm)	19 (19)	11(58)	08 (42)	01 (05)	18 (95)	02 (11)	17 (89)
T4 (any tumor size)	05 (05)	03 (60)	02 (40)	00 (00)	05 (100)	01 (20)	04 (80)
Lymph node status	100	57 (57)	43 (43)	13 (13)	87 (87)	16 (16)	84 (84)
Negative	51 (51)	30 (59)	21 (41)	06 (12)	45 (88)	09 (18)	42 (82)
Positive	49 (49)	27 (55)	22 (45)	07 (14)	42 (86)	07 (14)	42 (86)
Stage	100	57 (57)	43 (43)	13 (13)	87 (87)	16 (16)	84 (84)
Stage II	67 (67)	36 (54)	31 (46)	09 (13)	58 (87)	12 (18)	55 (82)
Stage III	33 (33)	21 (64)	12 (36)	04 (12)	29 (88)	04 (12)	29 (88)
Histopathology Type	100	57 (57)	43 (43)	13 (13)	87 (87)	16 (16)	84 (84)

Invasive Ductal Carcinoma (IDC)	77 (77)	45 (58)	32 (42)	10 (13)	67 (87)	12 (16)	65 (84)
Invasive Ductal Carcinoma + Ductal Carcinoma *in Situ*	10 (10)	08 (80)	02 (20)	02 (20)	08 (80)	01 (10)	09 (90)
Medullary Carcinoma	03 (03)	01 (33)	02 (67)	00 (00)	03 (100)	01 (33)	02 (67)
Papillary Carcinoma	03 (03)	00 (00)	03 (100)c	00 (00)	03 (100)	00 (00)	03 (100)
Lobular Carcinoma	05 (05)	03 (60)	02 (40)	01 (20)	04 (80)	01 (20)	04 (80)
Invasive Ductal Carcinoma + Mucinous Carcinoma	02 (02)	00 (00)	02 (100)c	00 (00)	02 (100)	01 (50)	01 (50)
Histology Grade (HG)	75	47 (63)	28 (37)	11 (15)	64 (85)	12 (12)	63 (63)
HG I	08 (11)	07 (87)	01 (13)	02 (25)	06 (75)	02 (25)	06 (75)
HG II and III	67 (67)	40 (60)	27 (40)	09 (13)	58 (87)	10 (15)	57 (85)
Bloom Richardson (BR) Score	80	45 (56)	35 (44)	11 (14)	69 (86)	12 (12)	68 (68)
Low (4-5)	14 (18)	08 (57)	06 (43)	01 (07)	13 (93)	01 (07)	13 (93)
Intermediate (6-7)	55 (69)	32 (58)	23 (42)	07 (13)	48 (87)	11 (20)	44 (80)
High (8-9)	11 (13)	05 (45)	06 (55)	03 (27)	08 (73)	00 (00)	11 (100)
Estrogen Receptor	100	57 (57)	43 (43)	13 (13)	87 (87)	16 (16)	84 (84)
Negative	63 (63)	40 (64)	23 (36)	09 (14)	54 (86)	10 (16)	53 (84)
Positive	37 (37)	17 (46)	20 (54)d	04 (11)	33 (89)	06 (16)	31 (84)
Progesterone Receptor	100	57 (57)	43 (43)	13 (13)	87 (87)	16 (16)	84 (84)
Negative	71 (71)	41 (58)	30 (42)	11 (16)	60 (84)	14 (20)	57 (80)
Positive	29 (29)	16 (55)	13 (45)	02 (07)	27 (93)	02 (07)	27 (93)
Her-2-Neu	100	57 (57)	43 (43)	13 (13)	87 (87)	16 (16)	84 (84)
Negative (score 0,+1)	59 (59)	35 (59)	24 (41)	07 (12)	52 (88)	09 (15)	50 (85)
Positive +2	18 (18)	08 (44)	10 (56)	03 (17)	15 (83)	04 (22)	14 (78)
Positive +3	23 (23)	14 (61)	09 (39)	03 (13)	20 (87)	03 (13)	20 (87)
Metastatic site	47	26 (55)	21(45)	09 (19)	38 (81)	07 (15)	40 (85)
Local recurrence	4 (9)	02 (50)	02(50)	01 (25)	03 (75)	01 (25)	03 (75)
Bone	17 (36)	11 (65)	06 (35)	04 (24)	13 (76)	03 (18)	14 (82)
Lung	10 (21)	07 (70)	03 (30)	01 (10)	09 (90)	01 (10)	09 (90)
Brain	02 (04)	02 (100)	00 (00)	00 (00)	02 (100)	00 (00)	02 (100)
Liver	05 (10)	01 (20)	04 (80)	01 (20)	04 (80)	01 (20)	04 (80)
Ovary	01 (02)	01 (100)	00 (00)	00 (00)	01 (100)	00 (00)	01 (100)
Multiple metastasis	08 (17)	02 (25)	06 (75)	02 (25)	06 (75)	01 (13)	07 (87)
Note: p value: a$\chi 2$ =4.93, r= 0.22, p= 0.02, b $\chi 2$= 2.94, r= 0.17, p=0.08, c $\chi 2$= 9.55, r= 0.15, p= 0.08, d $\chi 2$= 2.92, r= 0.17, p=0.08							

TABLE 2

***Aldh1a1, Cd133* and *Oct 4* expression in relation to survival.**

Marker Expression	N	DFS in months	Patients in Remission N (%)	Patients in Relapsed N (%)
		Mean ± SE		
***Aldh1a1* Expression**				
Negative	57	60.41 ± 4.15	31 (54)	26 (46)
Positive	43	53.97 ± 4.30	22 (51)	21 (49)
Log rank=0.19, df=1, p=0.65				
***Cd133* Expression**				
Negative	13	39.28 ± 7.11	09 (69)	04 (31)
Positive	87	62.41 ± 3.39	38 (44)	49 (56)
Log rank=3.43, df=1, p=0.06				
***Oct 4* Expression**				
Negative	16	56.57 ± 5.75	09 (56)	07 (44)
Positive	84	53.58 ± 3.51	44 (52)	40 (48)
Log rank=0.12, df=1, p=0.72				
Marker Expression	**N**	**OS in months**	**Alive N (%)**	**Dead N (%)**
		Mean ± SE		
***Aldh1a1* Expression**				

Negative	57	79.73 ± 2.88	46 (81)	11 (19)
Positive	43	71.34 ± 3.31	34 (79)	09 (21)
Log rank=0.17, df=1, p=0.67				
***Cd133* Expression**				
Negative	57	55.07 ± 6.38	09 (69)	04 (31)
Positive	43	81.27 ± 2.32	71 (82)	16 (18)
Log rank=2.68, df=1, p=0.10				
***Oct 4* Expression**				
Negative	16		16 (100)	0 (0)
Positive	84		64 (76)	20 (24)
Log rank=0.19, df=1, p=0.65				

TABLE 3

***Aldh1a1, Cd133* and *Oct 4* expression in relation to survival with respect to treatment**

Marker Expression	Treatment offered	N	Remission N (%)	Relapsed N (%)	Alive N (%)	Dead N (%)
Aldh1a1						
Negative	CMF and CMF with adjuvant therapy	6	06 (100)	00 (00)	06 (100)	00 (00)
	FAC and FAC with adjuvant therapy	49	23 (47)	26 (53)	38 (78)	11 (22)
Positive	CMF and CMF with adjuvant therapy	8	04 (50)	04(50)	06 (75)	02 (25)
	FAC and FAC with adjuvant therapy	34	17 (50)	17 (50)	27 (79)	07 (33)
			Log rank=0.21, df=1, p=0.64		Log rank=0.23, df=1, p=0.62	
Cd133						
Negative	CMF and CMF with adjuvant therapy	1	00 (00)	01 (100)	01 (100)	00 (00)
	FAC and FAC with adjuvant therapy	12	09 (75)	03 (25)	08 (67)	04 (37)
Positive	CMF and CMF with adjuvant therapy	13	04 (31)	09 (69)	11 (85)	02 (15)
	FAC and FAC with adjuvant therapy	71	37 (52)	34 (47)	57 (80)	14 (20)
			Log rank= 2.97, df=1, p=0.08		Log rank= 2.55, df=1, p=0.11	
Oct-04						
Negative	CMF and CMF with adjuvant therapy	3	03 (100)	00 (00)	03 (100)	00 (00)
	FAC and FAC with adjuvant therapy	12	05 (42)	07 (58)	12 (100)	00 (00)
Positive	CMF and CMF with adjuvant therapy	11	07 (64)	04 (36)	09 (82)	02 (18)
	FAC and FAC with adjuvant therapy	71	35 (49)	36 (51)	53 (75)	18 (25)
			Log rank= 0.03, df=1, p=0.84		Log rank= 3.08, df=1, p=0.07	
p value ≤ 0.05 is significant, df= degree of freedom						
CMF: Cyclophosphamide+Methotrexate+5-fluorouracil TMX: Tamoxifen FAC: 5-fluorouracil+Adriamycin+Cyclophosphamide RT: Radiotherapy						

significant difference was observed between *Cd133* protein expression and subgroups of age, menopausal status, tumor size, lymph node status, disease stage, histopathological subtypes, histological grade, Bloom Richardson (BR) score, ER, PR and *Her-2-neu* and metastatic sited in Table 1.

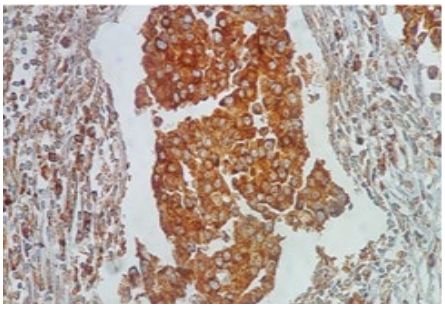

Figure 2) Membranous CD133 depicted by brown staining in tumor cells of breast cancer.

***Cd133* Expression in Relation to Survival:** Regarding Kaplan Meier univariate survival analysis with respect to DFS, a trend of higher incidence of disease relapse and reduced mean months DFS was noted in patients with negative *Cd133* expression (69%, 09/13; 39.28 ± 7.11 months) than patients with positive *Cd133* expression (44%, 38/87; 62.41 ± 3.39 months, Log rank= 3.43, df=1, p= 0.06; Figure 3). With respect to OS, a trend of higher incidence of death and reduced mean months OS was noted in patients with negative *Cd133* expression (31%, 04/13; 55.07 ± 6.38 months) than patients with positive *Cd133* expression (18%, 16/87; 81.27 ± 2.32 months, Log rank=2.68, df=1, p=0.10; Table 2).

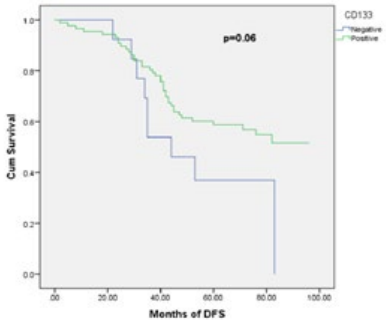

Figure 3) A shorter disease free survival was noted in patients with negative CD133 expression than patients with positive CD133 expression

***Cd133* Expression in Relation to Treatment:** In relation to treatment, patients with *Cd133* expression treated with FAC alone and FAC with

adjuvant therapy showed a trend of better DFS and OS than patients with negative *Cd133* treated with FAC alone and FAC with adjuvant therapy (Table 3).

***Oct 4* expression:** Nuclear *Oct 4* expression was observed in 84% of the tumors with H-score of 1+ in 05%, 2+ in 23% and 3+ in 56% (Figure 4). *Oct 4* protein expression when correlated with clinical and pathological parameters, significant difference was not observed between *Oct 4* protein expression and subgroups of age, menopausal status, tumor size, lymph node status, disease stage, histopathological subtypes, histological grade, Bloom Richardson (BR) score, ER, PR and *Her-2-neu*, and metastatic site (Table1).

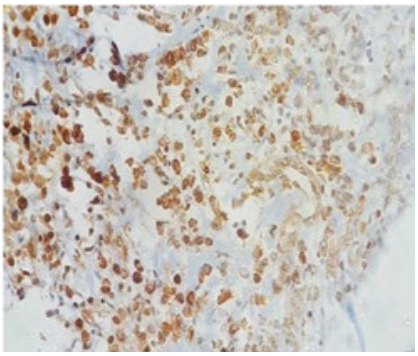

Figure 4) Nuclear Oct 4 depicted by brown staining in tumor cells of breast cancer.

***Oct 4* expression in relation to survival:** Regarding Kaplan Meier univariate survival analysis with respect to DFS, similar incidence of disease relapse and mean months DFS was noted in patients with *Oct 4* expression (48%, 40/84; 59.58 ± 3.51 months) and negative *Oct 4* expression (44%, 07/16; 56.57 ± 5.75 months, Log rank=0.12, df=1, p=0.72). With respect to OS, a trend of higher incidence of death was observed in patients with *Oct 4* positive expression (24%, 20/84) than patients with negative *Oct 4* (0%, 0/16, Log rank=3.43, df=1, p=0.06; Table 2 and Figure 5).

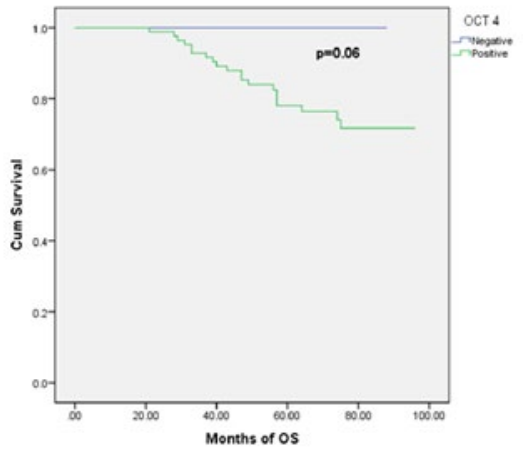

Figure 5) A shorter overall survival was noted in patients with positive *Oct 4* expression than patients with negative *Oct 4* expression.

***Oct 4* expression in relation to treatment:** In relation to treatment, patients with *Oct 4* expression treated with CMF alone and CMF with adjuvant therapy showed a trend of better OS than patients treated with FAC alone and FAC with adjuvant therapy (Table 3).

Regarding metastatic site, patients with liver metastasis and multiple metastasis showed all three stem cell marker expression in more than 75% of patients. Further, patients with brain and ovarian metastasis did not show *Aldh1a1* expression.

Intermarker correlation: Intermarker correlation of studied and previously studied EMT markers was performed. A significant positive correlation was observed between Oct 4 and *Cd133* expression ($\chi 2$= 15.92, r=0.39, p=0.002) and a significant inverse correlation was noted between *Cd133* and Vimentin expression ($\chi 2$=9.00, r= - 0.30, p= 0.003; Table 4).

Multivariate Survival Analysis: In multivariate survival analysis by Cox regression model with forward stepwise regression method, lymph node positive status entered at step 1 and *Cd133* negative expression entered at step 2 for predicting reduced disease free survival, and positive lymph node status entered at step 1, *Cd133* negative expression entered at step 2, and *Oct 4* positive expression entered at step 3 for predicting poor overall survival (Table 5).

DISCUSSION

This study identified breast cancer stem cells by combining all three markers in 35% patients with breast cancer. The *Aldh1a1* expression was observed in 43% of patients with breast cancer and it was observed in the range of 8.4% to 53% in studies on breast cancer [10-15]. Other than breast cancer, incidence of *Aldh1a1* expression was observed higher in lung cancer patients and lower in prostate and head-and-neck cancer [15-17]. Our study demonstrated a significant higher incidence of *Aldh1a1* expression in patients with age >45 years and a trend with histological type invasive ductal carcinoma + mucinous and papillary carcinoma, and ER positive status. There was no significant association of *Aldh1a1* expression obtained with lymph node, stage in this study which was in accordance to the study reported by Zhong et al. [18]. A study by Khoury et al. noted significant association of *Aldh1a1* with ER and PR negativity [19] and Mansour and co-workers [11,12,20,21] reported association that *Aldh1* with tumor size. Higher incidence of *Aldh1a1* expression was noted in histology grade II and III tumors in this study which was in accordance to the study of Yao et al. which showed a significant correlation between *Aldh1* expression and high histological grade [11]. *Aldh1a1* expression was seen higher in patients with liver metastatic patients of the present study. In a study by Zhong et al. *Aldh1* phenotype was seen to be associated with early recurrence [18]. With respect to disease status, it has been shown that *Aldh1 mRNA* and protein expression was associated with high recurrence rate and shorter DFS in breast cancer [5,10] and head neck cancer [17]. However, such a trend was not seen in our study with protein expression.

Cd133 protein expression in this study was found to be 87% while other studies have reported in the range from 40%-50% [20,21]. In non-small cell lung cancer and gastric adenocarcinoma it is reported to be 48.9% and 57.4% respectively. In our study significant association was not observed with age, lymph node, stage and tumor size. Contrary to that study by Sahar et al. obtained a significant correlation with age, lymph node, stage and tumor size [20]. Our study and study by Sahar et al. have not observed significant association of *Cd133* expression with histologic type [20]. A trend of higher incidence of *Cd133* expression was noted with advanced histology grade which was consistent with result obtained in a study by Han et al. [21]. With respect to disease status, a trend of *Cd133* negative expression with reduced disease free survival was observed in this study which was in accordance to another study which showed association between *Cd133* negative group and recurrence rates in colorectal carcinoma [22].

There are certain reports that suggest that *Oct 4* may serve the genesis of tumors because they play key role in maintaining the self-renewal capacity and pluripotency of embryonic stem cells and are biomarkers for cancer stem cells [23-27]. In present study, *Oct 4* protein expression was found to be 84%, while there are studies that have reported *Oct 4* expression in the range of 25% to 45% in breast cancer [25-27]. In present study *Oct 4* expression was not correlated with age and stage which was in accordance to the study of Dan Wang et al. [27]. Some studies reported significant association of *Oct 4* expression with lymph node status [25-29] which was not seen in our study. *Oct 4* expression was not correlated with tumor size and grade in our study while there are studies that contradict our results and shows significant association between tumor size and grade [26,27]. In this study a trend of *Oct 4* expression was associated with reduced disease free survival and overall survival. These findings are consistent with several studies which have shown significant association with disease free survival and overall survival [25,26,29,30].

Additionally these markers were correlated with EMT markers cytokeratin and Vimentin and observed a significant inverse correlation between *Cd133* and Vimentin expression which still has not been reported in any studies. Further, a significant positive correlation was observed between *Cd133* and *Oct 4* expression. Furthermore, multivariate analysis of the present study indicated lymphnode, *Cd133* as significant independent prognostic factor for predicting disease relapse while, lymphnode, *Cd133* and *Oct 4* as significant independent prognostic factor for predicting overall survival in breast cancer.

CONCLUSION

In conclusion, *Aldh1a1*, *Cd133* and *Oct 4* identified cancer cells with

TABLE 4

Inter-correlation of different markers

	Cd133 expression	Oct-04 expression	Cytokeratin expression	Vimentin expression
Aldh1a1				
r value	-0.5	-1.24	0.02	0.03
P value	0.8	0.54	0.79	0.71
Cd133				
r value		0.39	0.03	-0.3
P value		0.0001*	0.71	0.002*
Oct 4				
r value			-0.25	0.05
P value			0.8	0.6

TABLE 5

Multivariate survival analysis including all parameters

Patients	Step	Variables	Wald Statistic	df	p	Exp (B)	95% CI for Exp (B)	
							Lower	Upper
DFS N=47	1	Lymph node	16.82	1	0	5.72	2.48	13.16
	2	Cd133	4.26	1	0.03	0.4	0.17	0.95
OS N=20	1	Lymph node	7.72	1	0.01	8.2	1.86	36.18
	2	Cd133	4.12	1	0.04	0.29	0.09	0.94
	3	Oct-04	0.001	1	0.97	913664.8	0.0001	

stem cell phenotype and may have a role in initiation and progression of breast carcinoma. Also, *Cd133* and *Oct 4* could be served as an independent biomarker to predict worse prognosis in breast carcinoma patients.

Further *Cd133* may have a role in epithelial to mesenchymal transition. It can provide a drug target for molecular therapy for breast cancer.

CONFLICT OF INTEREST

There are no conflicts of interest to disclose from all authors.

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