Original Research

CD66 and CD49f expressing cells are associated with distinct neoplastic phenotypes and progression in human cervical cancer

Aswathy Ammothumkandy [a], Tessy Thomas Maliekal [a,e],
Mayil Vahanan Bose [b], Thangarajan Rajkumar [b], Sundersingh Shirley [c],
B. Thejaswini [d], Venkat G. Giri [d], Sudhir Krishna [a,*]

[a] *National Centre for Biological Sciences, Tata Institute of Fundamental Research, Bangalore, India*
[b] *Department of Molecular Oncology, Cancer Institute (WIA), Adyar, Chennai, India*
[c] *Department of Oncopathology, Cancer Institute (WIA), Adyar, Chennai, India*
[d] *Department of Radiotherapy, Kidwai Memorial Institute of Oncology, Bangalore, India*
[e] *Cancer Research Program, Division of Cancer Research, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, India*

Abstract *Background:* In this study, building on our recent work identifying a subset of CD66+ve cells with distinctive tumourigenic properties in human cervical cancers, we examine patterns of expression and function of these cells; to generate insights into the process of metastasis.
Methods: Our broad approach in this study has been to compare the expression and function of two subsets marked by CD66 and CD49f. We use a combination of histopathology, immunostaining and flow cytometry, functional analysis of an established cervical cancer cell line and a retrospective analysis of a cohort of cervical cancer.
Results: We noted CD66 expression associated with clusters of cells which are spindle shaped, SMA+ve, podoplanin+ve, phalloidin high, fibronectin high, plakoglobin low, ki67−ve and CK10+ve at the migratory phase along with features of partial EMT. Further, TGFβ1 a well known regulator of EMT, positively correlated with CD66 expression. The additional CD49f+ve subset at the leading invading front of migration was SMA−ve, phalloidin low, fibronectin low, plakoglobin high, Ki67+ve and CK14+ve. These data are consistent with a

* *Corresponding author*: Sudhir Krishna, National Centre for Biological Sciences, TIFR, UAS-GKVK Campus, Bellary Road, Bangalore, 560065, India. Tel.: +91 80 23666070; fax: +91 80 23636662.
E-mail address: skrishna@ncbs.res.in (S. Krishna).

role for CD66 cells in metastatic invasion with a collective cell migration process co-opting the CD49f subset. Our retrospective analysis of a cohort is consistent with a role for CD66 in metastasis. However, the broad analysis of CD66, CD49f and TGFβ1 expression with patterns of overall survival points to a possible protective effect particularly for local recurrences. Hence, future studies focussing on potential heterogeneity within the CD66 subset along with the possible role of isoforms and intra-cellular roles would be essential.

1. Introduction

An ongoing challenge in cancer biology is to understand how distinct tumourigenic subsets mediate progression of human cancers [1]. In particular, the mechanisms and contribution of Epithelial to Mesenchymal Transition (EMT), the linkage with chemo resistance, and the stage specific role of such subsets are currently poorly understood phenomena. The CD66 gene is a part of the carcinoembryonic antigen related cell adhesion molecule (CEACAM) family with various splice variants, amongst which CD66a, c and e are highly expressed in several cancers and have been suggested to mediate poor outcomes in some instances [2–5]. We have recently characterised a subset of CD66+ve cells in cancers [6] and pre-cancers [7] of cervix with distinctive tumourigenic properties. While invasiveness of CD66+ cells in both cervical pre-cancer and cancers seems a hallmark of this subset, there are parallel complex observations on the differentiation and cell cycle status of this subset which has made the generation of simplistic models of tumourigenic progression somewhat difficult. Recent studies on breast cancer progression draw attention to the need to look at multiple subsets, and possibly gene expression along with differentiation in the setting of collective cell migration [8–10], as this might be pathophysiologically more relevant.

The other specific subsets in human cervical cancer that have been identified include CD49f+ cells, which have been suggested to be tumour initiating cells by some investigators [11–13]. CD49f is expressed in the basal layer of normal cervix and is present even in the upper layers of ectocervical epithelium in cervical intraepithelial neoplasia [14]. CD49f and its interacting partners have also shown to have an association with invasion [15–21] across tumours and is a plausible marker for a basal cell that can lead collective invasion. In addition to analysing the role of specific subsets, it is critical to examine putative signalling modulators, such as TGFβ. TGFβ has shown to have both growth inhibiting and EMT inducing roles [22,23] in a range of cancers. From other reports in cervical cancer, TGFβ is associated with less tumour infiltration and a tumour suppressor function [24,25].

In a clinico-pathological perspective of collective cell migration, tumour sections have shown presence of sarcomatoid cells, with the suggestion that these cellular clusters drive metastasis and drug resistance [26,27]. Such sarcomatoid cells are infrequently found in human carcinomas and appear as bundles of spindle like or giant multinucleated cells marked by the expression of smooth muscle actin (SMA) and podoplanin. In addition, they have features of partial EMT i.e. expressing both E and N-cadherin, often referred to as metastable cells [28].

In this study, we analysed the expression of CD66 in sarcomatoid like clusters of cells in primary human cervical cancers along with CD49f+ cells. We developed assays for collective invasion of a human cervical cancer cell line, and also assessed the role of CD66/CD49f subsets. Following the characterisation of sarcomatoid cells, we explored the role of TGFβ1 and its association with CD66 expression and possible metastatic outcomes. In addition to a functional analysis of an established human cervical cancer cell line, we examined CD66, CD49f and TGFβ1 expression in a cervical cancer patient cohort with known clinical outcomes. The study attempts to address the question of whether there is possible heterogeneity in the CD66+ subset in terms of clinical significance, as this would lay the foundation for an analysis of CD66 isoforms, intra-cellular roles, possible stage specific roles linked to differentiation etc.

2. Materials and methods

2.1. Collective invasion assay with spheres in matrigel

The cell line CaSki, obtained from ATCC was cultured as spheroids for 14 days as previously described [6]. Spheroids were collected in sphere media, mixed with equal amount of matrigel and seeded in confocal dishes such that spheroids were spaced far apart. Sphere media was added after the matrigel solidified. After 30 hrs, cells were fixed with 4% PFA for 20 min. 3 PBS washes were given for 10 min each. Regular IF protocol was followed with all incubation time points 3× times.

2.2. Clinical sample processing

Primary human cervical SCC biopsy samples processed as previously described [6]. Cells were lineage depleted
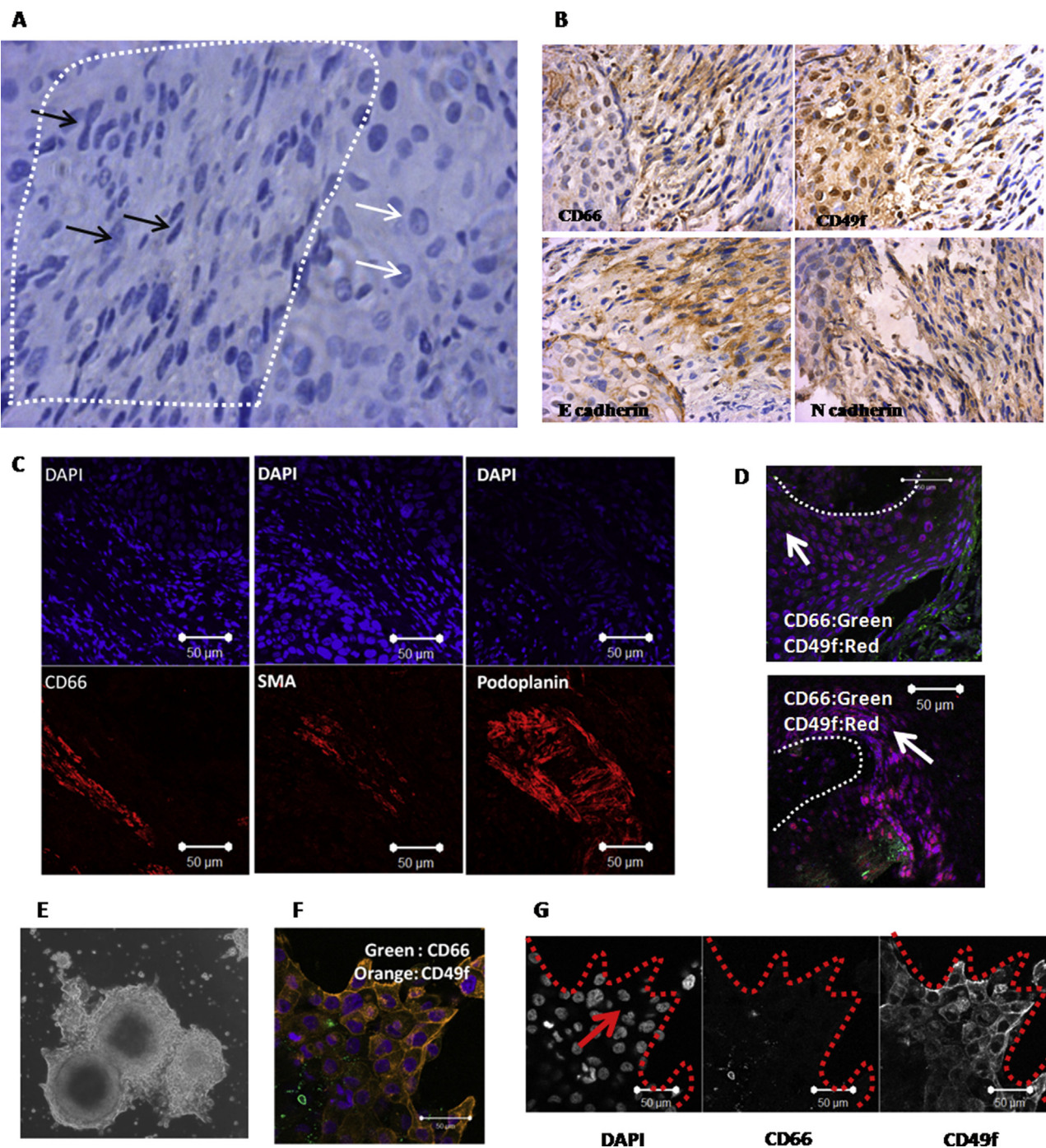
Fig. 1. **CD66 expression is associated with collectively invading tumour clusters and CD49f at the invasive front in human cervical cancer.**
A) C13 tumour section shows cells with sarcomatoid morphology (encircled with dotted lines). The sarcomatoid cells have either spindle shape morphology or giant multi-nuclei (black arrows) compared to the typical rounded carcinoma cells (white arrows). 40× Magnification B) CD66, CD49f, E-cadherin and N-cadherin expression in spindle and rounded cells at 40× magnification. Sarcomatoid like phenotype was observed only in 2/11 cases examined. The serial sections of these sarcomatoid like cells show higher expression of both epithelial marker E-cadherin and mesenchymal marker N-cadherin. C) Serial sections of tumour with sarcomatoid cells show that the CD66+ve sarcomatoid cells are positive for SMA and podoplanin. D) Staining of CD66 and CD49f in sections with early phases of invasion into the basal lamina shows CD49f+ (red) at the invading edge and CD66+ (green) cells present in the differentiated top layers. The dotted line separates the epithelial and dermal layer. The arrow denotes the direction of invasion (n = 4). Scale bar 50 μm. E) CaSki-spheroids embedded in matrigel invade collectively with finger-like protrusions. F, G) Grey scale and overlapping images showing the invasion fingers express CD49f (orange) at the leading front and CD66 (green) at the trailing cells. Dotted line shows the outer boundary for invading cells .Scale bar– 50 μm. Figure F, G are representative images from a minimum of 3 independent experiments.

cells using MACS column, stained for proteins and analysed by FACS. Biopsy samples were labelled as C − a number (in serial order).

3. Results

3.1. CD66 expression is associated with collectively invading tumour clusters and CD49f at the invasive front in human cervical cancer

We examined the expression of CD66 and CD49f in the context of invading cells in primary cervical cancer. We searched for clusters of cells that may be putative sarcomatoid cells in cervical squamous cell carcinoma (SCC's) (n = 11). Sarcomatoid like cells were identified in 2/11 cases with the typical morphology of spindle like cells in collective bunches with few giant nuclei (Fig. 1A). We further undertook immunostaining of these cells for CD66 and CD49f along with some of the markers known to be co-expressed in a typical sarcomatoid cell − E-cadherin, N-cadherin, SMA and podoplanin (Figs 1B,C, and S1A). These clusters were E-cadherin, N-cadherin, SMA, podoplanin and CD66+ve.They expressed lower levels of CD49f compared to normal carcinoma cells. Thus sarcomatoid like cells have features of partial EMT, consistent with a role in metastatic progression.

To examine CD66 and CD49f in the context of collective cell migration, we examined regions that are likely to represent the initial invasive phase in primary SCC's along with CaSki cells undergoing collective cell migration. CaSki-spheroids are heterogeneous for CD66 and CD49f expression with around 20, 14, 7, 59 percentages of CD66+CD49f−, CD66−CD49f+, CD66+CD49f+ and CD66−CD49f− subsets respectively in a 14 day spheroid culture (Fig. S1B). We assessed the invasive cell clusters which were prominent after 30 hrs of culturing CaSki spheroids in matrigel (Fig. 1E). The invasive cells expressed both epithelial marker E-cadherin and mesenchymal markers N-cadherin and vimentin (Fig. S1C). In the context of both initial invasive phases of SCCs (Fig. 1D) and CaSki invasion assay, CD49f cells are ahead of CD66 cells in the advancing invasive zone (Fig. 1F,G). These data show that CD49f expressing cells are present at the invasive front of collectively invading CD66+ve tumour clusters.

3.2. CD49f and CD66 subsets associated with distinct neoplastic traits

Having noted that CD66 and CD49f expression is segregated into different cells in invading clusters, we explored their functional traits. We use cytokeratin and ki67 staining to assess the basal and proliferative potential of these cells. The features of migration and EMT

were evaluated by staining for phalloidin, SMA, fibronectin and plakoglobin. These markers were evaluated along with CD66 and CD49f in CaSki invasive protrusions. CK14 (basal) and CK10 (differentiation) expression was present at the leading and trailing compartments respectively (Fig. 2A). A greater number of ki67+ve cells were present in the leading front (Fig. 2B) compared to the trailing region. Trailing cells were marked by high phalloidin (Figs. 2C and S2), high fibronectin and low plakoglobin expression (Fig. 2D). As we find enhanced features of EMT and migration in the trailing cells, we proposed the leading edge to be more like a growing/proliferating edge of the advancing tumour. In order to look at spatiotemporal progression, we also undertook phalloidin and CD49f staining at the zones proximal to CaSki spheroids which are regions of likely initiation of invasion (Fig. 2E). We find increased expression of CD49f in these regions along with stronger phalloidin staining as the cells initially invade out of spheroid (Fig. 2E,F).

Further, tumours with features of early invasion show higher expression of SMA in differentiated trailing edges in comparison to leading edges (Fig. 2G). Further, the basal regions of stratified epithelium had greater numbers of ki67+ve cells in both normal cervix (Fig. 2H) and in SCC sections (Fig. 2I). Similarly, expression of CD49f was high in the basal region of cervical neoplasms and was absent towards differentiated regions (Fig. 2J). Further, we looked at sarcomatoid cells in SCC and observed that these cells are CD66+ve and ki67−ve, whereas non sarcomatoid tumour cells around are CD66−ve and Ki67+ve (Fig. 2K). Collectively, our observations in Fig. 2 show that both in primary tissue and cervical cancer cell line model, the leading CD49f positive cells are more basal and proliferative; whereas trailing CD66 cells are more differentiated, migratory and show features of EMT. We have previously shown[7] using dye-dilution experiments that in whole CaSki-spheroids, CD66−CD49f+cells proliferate more compared to other subsets. Also we have shown [7] that CD66 and CD49f subsets follow a hierarchy in the expression of differentiation markers S100P and Involucrin in sorted cells from cell line and patient samples. CD66+CD49f− cells express highest levels of S100P and involucrin, followed by CD66+CD49f+ cells and then the CD66−CD49f+ve cells.

3.3. CD66 and CD49f subsets from entire tumours also show distinctive traits

In Figs 1 and 2, we used histopathology on invasive zones and demonstrated the distinct localisation and function of CD66 and CD49f subsets. We further evaluated if the properties of CD66 and CD49f cells are retained in the bulk of the tumour cells. We measured proliferation (by Ki67) and EMT associated markers in
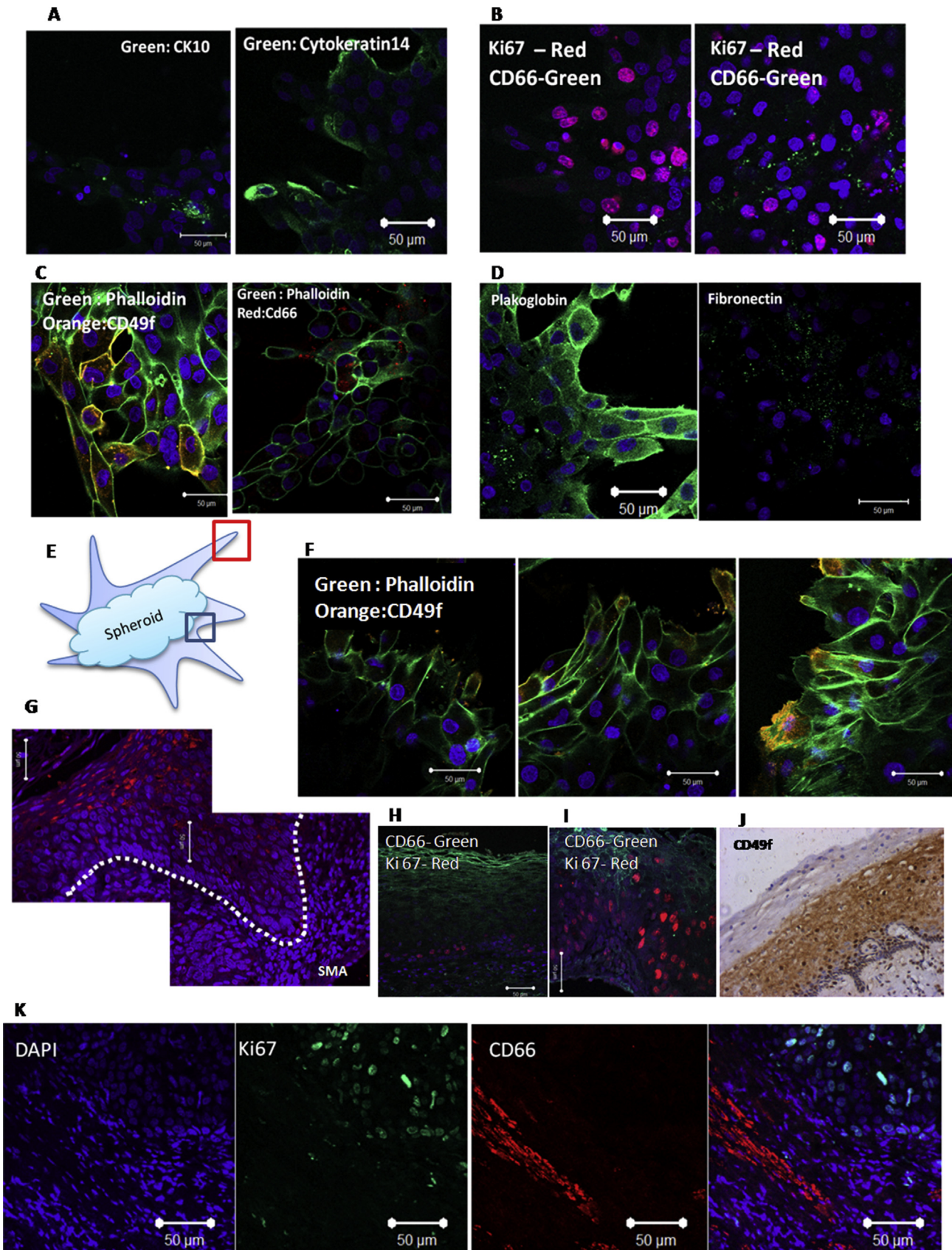
Fig. 2. **CD49f and CD66 subsets associated with distinct neoplastic traits.** A) The leading edge outer cells show expression of high basal associated Cytokeratin CK14 (green) and low differentiation associated Cytokeratin CK10 (green) in contrast to trailing cells. B) The leading edge has more ki67+ve cells (red) a marker for proliferating cells compared to trailing cells which express CD66 (green). C) The

CD66 and CD49f subsets from CaSki spheroids or tumour biopsies using flow cytometry. Ki67 and Oct4 (pluripotency marker) high cells from CaSki-spheroids showed higher expression of CD49f (Fig. 3A,B). We also observe that the chemotherapeutic drug-Cisplatin reduces the proliferative CD49f+ve pool from CaSki spheroids (Fig. 3C). The analysis of CaSki-spheroids (Fig. S3A,C) and lineage-depleted tumour cells from patient biopsies C50 and C51 (Figs 3D,E, and S2B,D) reveal that CD66+ve cells from bulk tumour cells have features of partial EMT, measured by higher expression of all the three proteins − E-cadherin, N-cadherin and vimentin. Collectively from Figs. 1,2,3A−E, we find a striking correlation of CD49f and CD66 subsets with proliferation and partial EMT respectively.

Having characterised the functional properties of CD66 and CD49f subsets, we proceeded to explore their possible clinical implications. We evaluated the inter-tumour heterogeneity of CD66 and CD49f expression from primary cancers. Biopsies of primary cervical cancer showed different ratios of CD66 and CD49f when analysed by flow-cytometry at the time of first clinical presentation (Fig. 3F). There is an intriguing association of CD66 expression (Fig. 3F) and the presence of sarcomatoid cells (2 cases with sarcomatoid cells mentioned in Fig. 1A are samples C13 and C14). The extensive heterogeneity of CD66 and CD49f expression led us to search for possible signature cues which would induce these phenotypes.

3.4. TGFβ, an EMT signaling pathway is associated with higher CD66 to CD49f subset ratios

TGFβ1 has been previously shown to induce EMT in wide range of human cancers. We therefore explored the link between CD66 and CD49f expression with TGFβ1 levels. We analysed the expression of TGFβ1 and a key downstream intermediate pSmad3 in SCC sections (Figs 4A and S7) and compared with the CD66 and CD49f expression in the same samples as analysed by FACS (Fig. 3F). Across patient samples the tumours (C13) with highest fraction of CD66+CD49f− subset (Fig. 3F) shows increased levels of TGFβ1 and nuclear pSmad3 expression (Fig. 4A). CD66+ve subset showed a clear correlation with TGFβ1 score (Fig. S4A). CD66+CD49f− subset had a stronger positive correlation (Fig. 4B) with TGFβ1 score. Consistent with these

results, in vitro induction of EMT in CaSki-spheroids by TGFβ1 treatment for 72 hrs increased the CD66+ CD49f− subset and decreased CD66−CD49f+ subset (Figs 4C and S4C). There was a concomitant increase in migration property (Fig. 4D) as measured in a transwell assay. TGFβ1 treatment also induced a cell proliferation arrest as measured by WST-cell proliferation assay (Fig. S4B) and clonogenic assays (Fig. 4E). Collectively the data from Fig. 1−4 show a consistent association of CD66 subset migration and posit TGFβ1 as a potential inducer.

3.5. Human cervical cancer metastasis is associated with high CD66, TGFβ1 and CD49f expression

We further looked at association of CD66 and CD49f in reported cases of lymph node metastasis. The staining suggested that all the 4/4 cases with lymph node metastasis expressed high levels of CD66 and CD49f in the primary tumour site (Fig. 5A). To further analyse subsets that are enriched in the lymph node metastasis we performed CD66 and CD49f staining in their respective matched lymph node tumour section. In all the 4 cases the expression of CD66 and CD49f was high and also they followed a similar pattern as that of primary tumour (Figs 5B and S8). This data is consistent with a functional role for CD66 in metastasis with a possible supportive role for CD49f+ve cells. In Table 1 we show a similar pattern of high CD66, TGFβ1 and CD49f scores associated with distant metastatic outcomes observed in a different larger cohort study. Collectively high CD66 and CD49f expression is associated with both lymph node and distant-metastasis. Our model of cervical cancer invasion in which distinct tumour subsets CD66 and CD49f with properties of migration and proliferation respectively driving tumour progression is presented in the graphical abstract.

3.6. CD66 and TGFβ 1 positive tumours are associated with better overall survival

We were further interested in two aspects of CD66 expression in terms of clinical outcomes- whether the diverse clinical outcomes correlate with CD66 expression and if it can be measured by broad CD66 expression in tumour sections. The cohort consisted of 153

---

trailing CD66+CD49f− cells (red) are high for phalloidin (green) staining and leading edge CD66−CD49f+ cells (orange) express lower levels of phalloidin. D) The trailing cells show features of EMT like high fibronectin and low plakoglobin, compared to leading cells. E) Representative image of spheroid invasion assay. Red box denotes the region of invasive protrusion studied in Fig. 2A−D, Blue box denotes zones closer to spheroid where cells start to invade. F) CD49f and phalloidin staining in zones closer to spheroid showing an increase in CD49f expression as phalloidin fibers start to appear. The Fig. 2A−F are representative images of minimum 3 independent experiments. Scale bar − 50 μm. G) Early invasions in SCCs show expression of SMA (red) in differentiated trailing end. Dotted line separates the dermal and epidermal layer. H, I) Cervical stratified epithelium shows more of ki67+ve cells towards the basal layers of H) normal cervix and I) SCC. J) Basal region of stratified epithelium shows higher CD49f expression. The Fig. 2G−J are representative images of observations from a minimum of 4 different patient sections. K) CD66+ve sarcomatoid cells in SCC sections are ki67−ve.
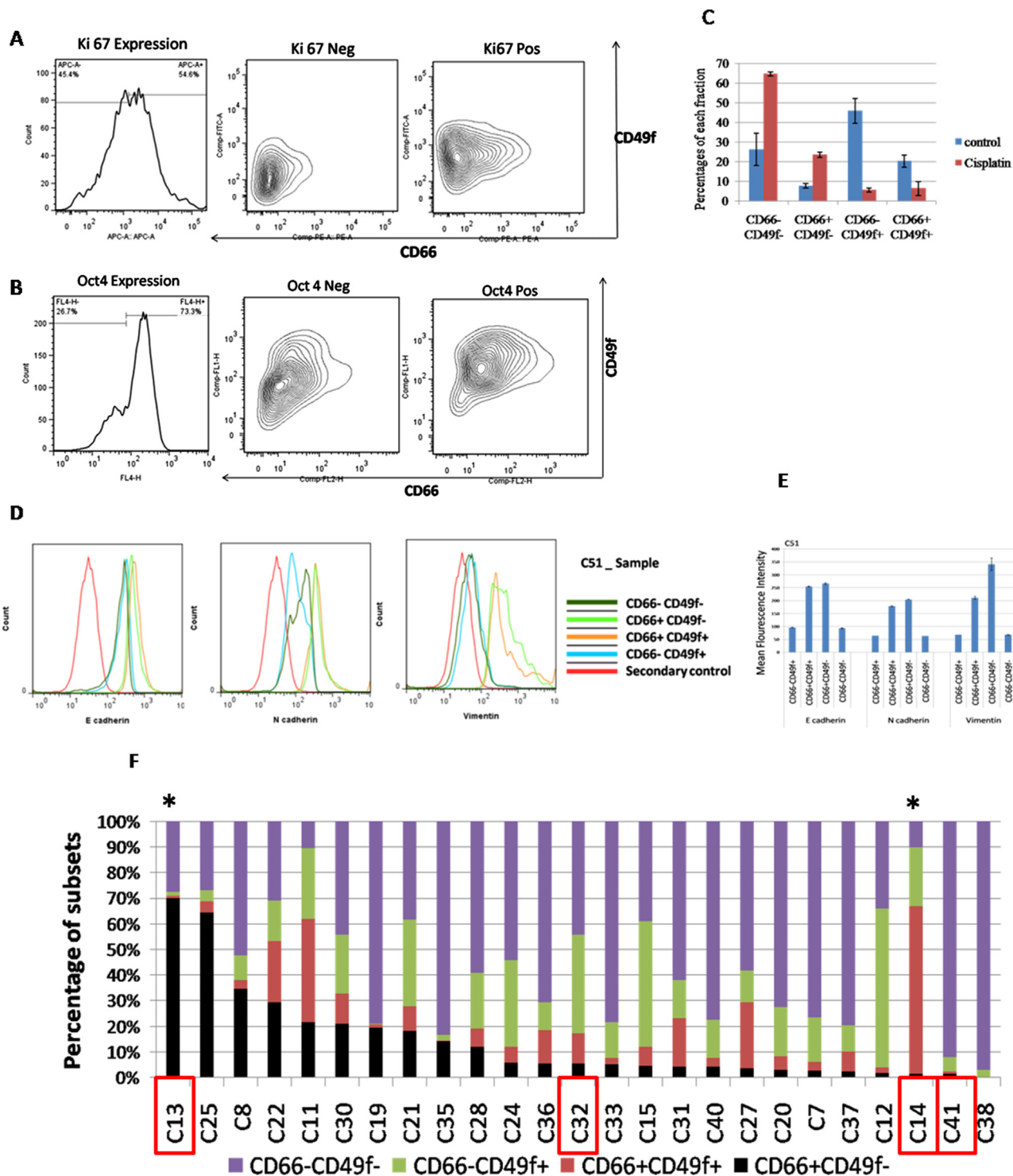
Fig. 3. **Properties and ratios of CD66 and CD49f cells in the bulk tumour.** A) Ki67 high cells in CaSki spheroids are CD49f high. B) Oct4 high cells in CaSki spheroids are CD49f high. (A, B. Representative plot minimum of 3 biological replicates in CaSki spheroids). C) Percentage of CD66 and CD49f fractions with and without Cisplatin treatment for 24 hrs on CaSki 14 day spheroid (n = 3). D) CD66+CD49f+ and CD66+CD49f− subsets in primary cervical cancer biopsies express both epithelial (E-cadherin) and mesenchymal markers (N-cadherin and vimentin) at a higher level as shown by histograms derived from FACS analysis and E) quantification of mean fluorescence intensity of E, N-cadherin and vimentin across CD66 and CD49f subsets. Error bars correspond to n = 3 technical replicates of the same patient. F) Frequency analysis of CD66 and CD49f subsets by flow cytometric analysis in cells isolated from primary cervical cancer biopsy after lineage depletion (n = 25). Samples marked with * on the top are the 2 samples with sarcomatoid cells mentioned in Fig. 1A. Sample names boxed with red are stained for TGFβ1 and pSmad3 in Fig. 4
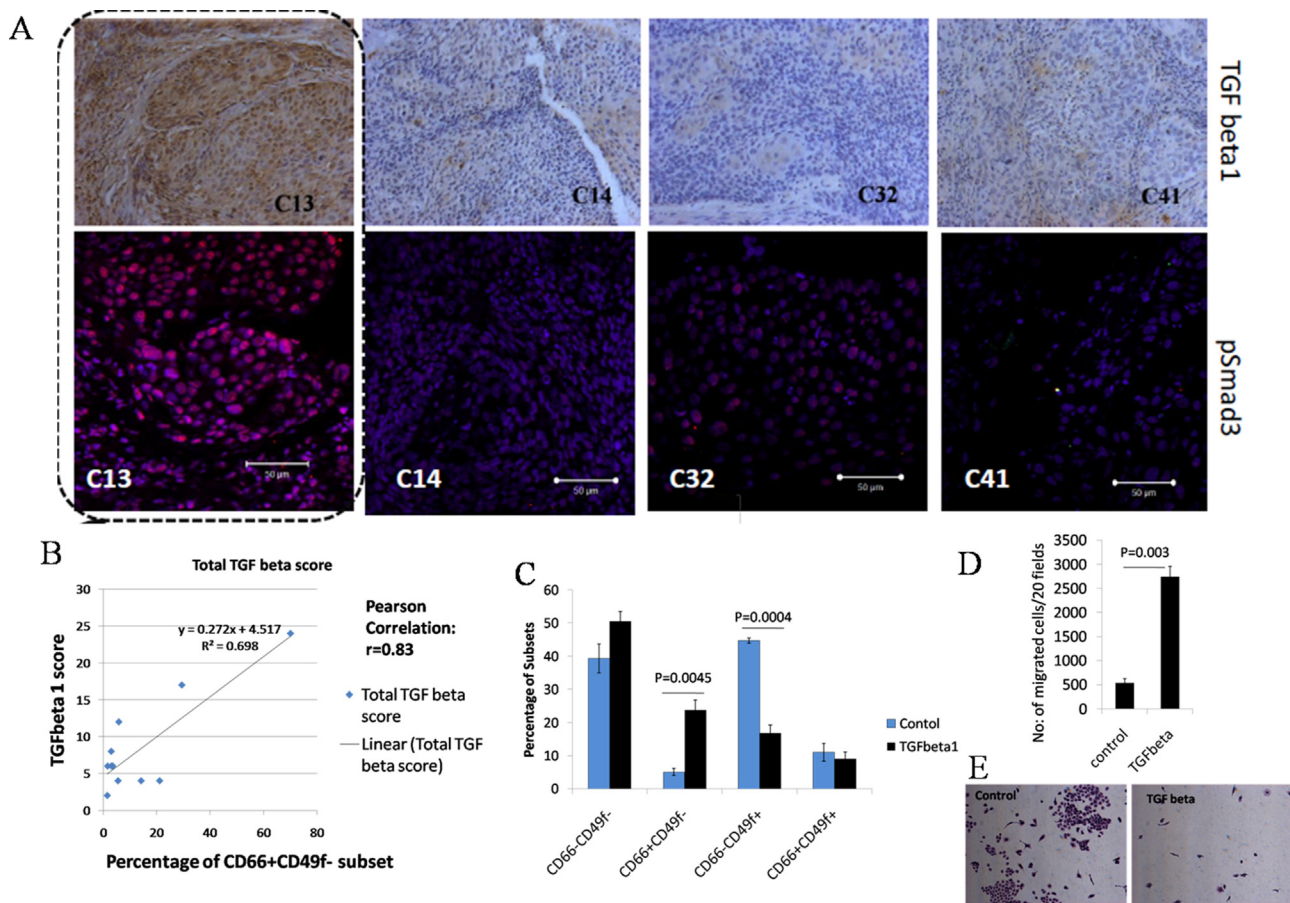
Fig. 4. **TGFβ, an EMT signalling pathway is associated with higher CD66 to CD49f subset ratios.** A) TGFβ1 and pSmad3 expression by IHC in representative patient samples show greater level of both these proteins in C13 sample which has the highest percentage of CD66+CD49f− subset. TGFβ1 staining is performed in n = 11 patient sections and pSmad3 in n = 6 patient sections. The images shown are from patients C13, C14, C32, C41 which are from biopsies having high percentages of CD66+CD49f−, CD66+CD49f+, CD66−CD49f+, CD66−CD49f− subsets respectively as shown in Fig. 3D (boxed in red). Scale bar 50 μm. B) The percentage of CD66+CD49f− subset in primary cervical cancer biopsies (n = 11) as analysed by flow cytometry shows positive correlation with TGFβ1 IHC score. Pearson's correlation = 0.83, p = 0.001362. C) Treatment of CaSki-spheroids with 2 ng/ml TGFβ1 growth factor for 72 hrs expands the CD66+CD49f− subset and reduces the CD66−CD49f+ subset. Minimum 3 biological replicates. D) Treatment of CaSki-spheroids with TGFβ1 increases the migration potential of these cells as measured by a Boyden chamber assay. Experiment biologi-cally replicated twice with 3 technical replicates. p value in Fig. 4C and D determined by student's t test, and two tailed. E) CaSki spheroid cells in clonogenic assay after 10 days shows an arrest in cell proliferation with continuous TGFβ (2 ng/ml) treatment. Representative image from 3 biological replicates.

cervical cancer SCC cases that underwent prescribed treatment and had a follow-up of 7 years or more. Of the 153 patients studied, 55 cases failed in the primary line of treatment, principally radiotherapy. Out of the 55 cases with poor outcome, 7 developed distant-metastasis (Table 1) and 36 had either partial response with treatment followed by progression or developed local relapse after achieving apparent complete clinical remission (Fig. 6A). In addition, there were 12 cases with unknown cause of death all of whom were pre-sumed to have been lost to disease. This data is consistent with the clinical paradigm that loco-regional failure is the major cause of poor clinical outcomes in cervical cancer.

We analysed the immuno-histochemical expression of CD66, CD49f and TGFβ1 and correlated it with clinical outcomes (Fig. 6 and Table 2).Patients with TGFβ1 expressing tumours had the best outcome with 91/133 being free of disease, while amongst the TGFβ negative tumours 7/20 only were free of disease (Fig. 6E,H). Patients with CD66+ tumours had better outcome compared to CD66− tumours (Fig. 6B). Of the 115 CD66+ cases 79 were disease free compared to 19/38 cases being disease free in the CD66− cases. CD49f+ and CD49f− tumours showed similar survival pattern (Fig. 6C). TGFβ in association with CD66 or CD49f was found to predict a better outcome albeit at a lower significant level, suggesting that the main effect was from TGFβ expression (Fig. S6). CD66, CD49f dual
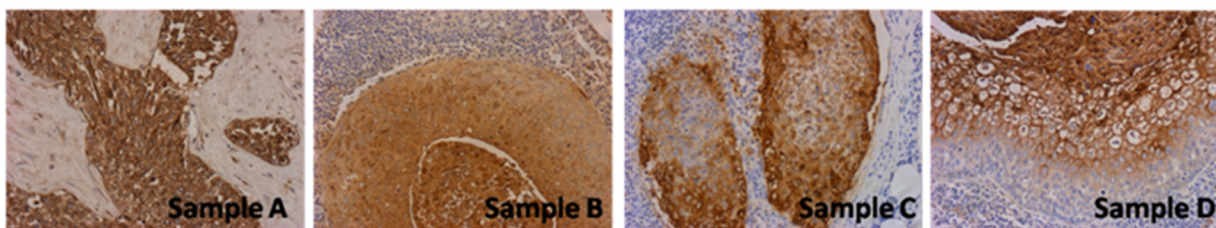
A Primary tumor CD66 expression
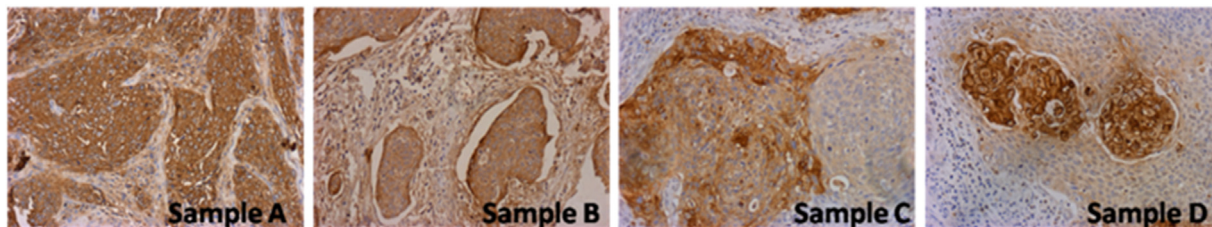
Primary tumor CD49f expression
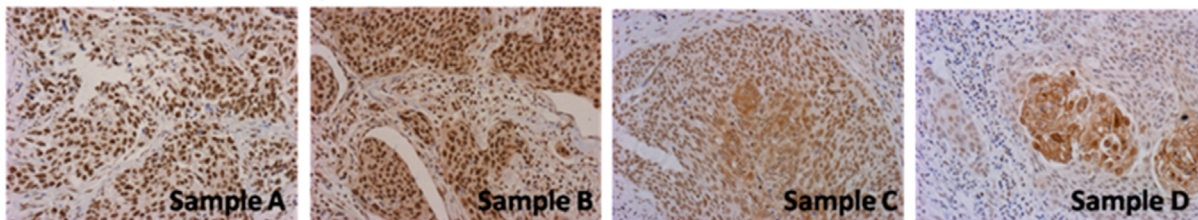
B

Metastatic tumor CD66 expression
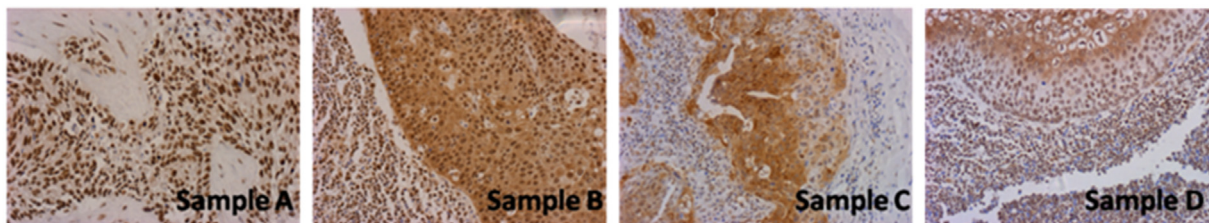

Metastatic tumor CD49f expression

Fig. 5. **Human cervical cancer metastasis is associated with high CD66, TGFβ1 and CD49f expression.** A) Primary biopsies for tumours which showed lymph node positive metastatic tumours have high expression of both CD66 and CD49f in all the 4 cases studied. B) Matching lymph node sections for these tumours also show high expression of CD66 and CD49f.

positive cases showed better DFS and OS compared to other permutations of expression (Fig. 6D,G). Concomitantly, the CD66, CD49f, TGFβ1 triple positive cases also had a better survival advantage (Fig. 6F,I). DFS data is included in the supplementary (Fig. S5,S9). In early stage cervical cancer (n = 18), none of the CD66/CD49f double positives (n = 9) failed to respond to treatment, whereas lack of expression of either CD66 or CD49f (n = 9), had 4 failures. In late stage CD66/CD49f double positives tumours 28/86 where associated with failures (Fig. 6J). Overall CD66 and TGFβ1 positive tumours (Fig. 6B,E) were associated with better overall survival possibly due to the bias imposed by

Table 1
Scores for CD66, CD49f and TGFβ in all the cases of distant-metastasis in retrospective study

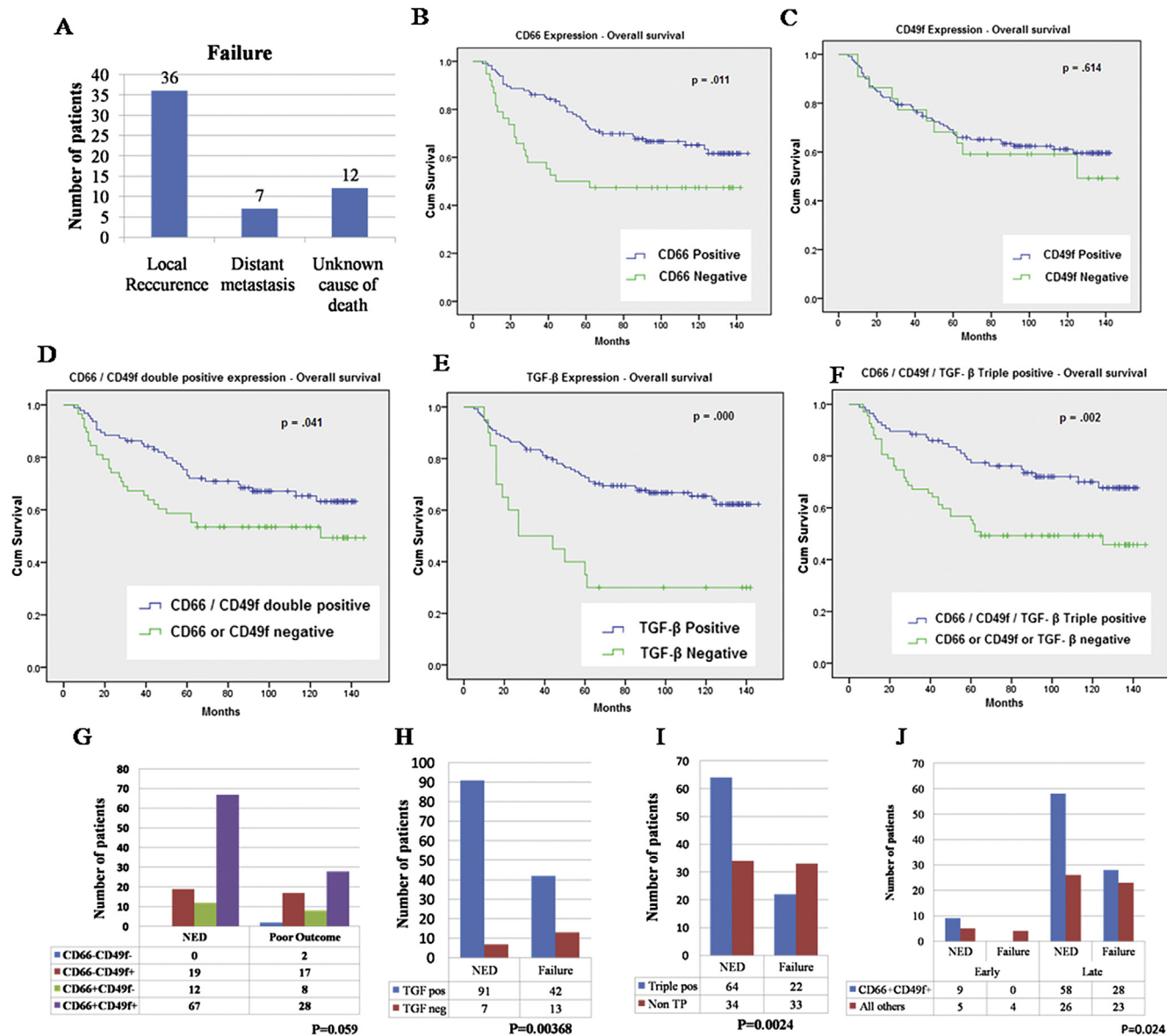| Biopsy no.	CD66 score	CD49f score	TGF beta1 score	Disease free survival	Overall survival	Metastasis
4249/02	5	6	5	50	113	Lung and liver
5432/02	6	4	4	13	20	Lung
6562/02	4	7	3	5	5	Lung
4526/02	4	7	4	39	46	Bone
5558/02	0	7	0	6	16	Bone
892/02	5	6	5	31	38	Lung
1015/02	6	0	4	43	65	Lung

Fig. 6. **CD66 and TGFβ 1 positive tumours are associated with better overall survival.** A) The patients with poor outcome belong majorly to local-recurrence. B−F) Kaplan−Meier curves for overall survival (OS) in SCC of cervix (N = 153) shows: B) according to CD66 expression status alone, statistically significant increase in OS was observed in CD66 positive tumours (p = .011). C) According to CD49f expression status, no statistically significant increase in OS was observed in CD49f positive tumours (p = .614). D) According to CD66/CD49f double positive expression status, statistically significant increase in OS was observed in CD66/CD49f double positive tumours (p = .041). E) According to TGF-β expression status, statistically significant increase in OS was observed in TGF-β positive tumours (p = .000). F) According to CD66/CD49f/TGF-β triple positive expression status, statistically significant increase in OS was observed in CD66/CD49f/TGF-β triple positive tumours (p = .002). G−I) Distribution of outcome and expression in 153 patients and their significance with chi square test: G) CD66/CD49f dual expression tends to correlate with No evidence of disease (NED) compared to when expressed alone (p = .059). H) TGFβ expression correlates with more chances of NED (p = .004). I) CD66/CD49f/TGFβ triple positives have higher correlation with NED (.002). J) Distribution of outcome and expression for CD66 and CD49f in early versus late stage tumour (p = .024).

Table 2
Cox prognostic factors by vital status (Overall survival) CD66, CD49f and TGFβ1 in 153 cases of human cervical SCCs.

Cox prognostic factors by vital status (Overall survival)								
			Univariate analysis			Multivariate analysis		
Variables	Factors	Cases	HR	95% CI	p Value	HR	95% CI	p Value
CD66	Positive	115	1.00[a]			1.00[a]		
	Negative	38	1.976	1.154 − 3.385	.013*	1.991	1.142 − 3.471	.015*
CD49f	Positive	131	1.00[a]			1.00[a]		
	Negative	22	1.19	.604 − 2.347	.615	1.288	.627 − 2.644	0.491
TGFβ1	Positive	133	1.00[a]			1.00[a]		
	Negative	20	2.88	1.576 − 5.264	.001*	2.536	1.373 − 4.682	.003*
CD66 and CD49f double positive	Positive	95	1.00[a]			1.00[a]		
	Negative	58	1.686	1.015 − 2.801	.044*	1.708	1.025 − 2.847	.040*
CD66,CD49f and TGFβ1 positive	Positive	86	1.00[a]			1.00[a]		
	Negative	67	2.205	1.318 − 3.688	.003*	2.179	1.300 − 3.653	.003*

[a] Reference category.
* = p < 0.05.

4. Discussion

In this study, we have addressed three inter-related questions i) Given the focus on migration from previous studies on CD66+ cells, what are the mechanistic insights that emerge on a more detailed analysis of this particular function? ii) Are there distinctive expression profiles and concomitant functional properties for CD66+ and CD49+ cells iii) Do the clinical outcomes assessed largely based on expression profiles of CD66 provide insights into the emergence of both metastasis and local recurrence ?

In this study, we identify clusters of CD66 cells which based on the features of partial EMT, migration, morphology and non-proliferative state that are consistent with a role in metastasis. Such sarcomatoid cells are difficult to detect across cancers and their mechanistic role in driving human tumour progression is of enormous interest. Our observations in this study are consistent with more complex models of migration being associated with tumour progression in the form of collective cell migration [29−31]. In particular, our data reveals two distinct CD66 and CD49f subsets in both primary cancers and established cell lines and imply that therapeutic targeting might require a synergistic approach to address issues of proliferation and migration simultaneously. This is likely to be further complicated by the plasticity that we also potentially detect in the emergence of CD49f subsets over the various phases of collective cell migration (Fig. 2F). Our observation of CD66−CD49f+ leader cells is in keeping with the literature which posits basal cells at the leading edge [8]. However, with respect to migration, contrary to other studies the leading edge CD66−CD49f+ cells have reduced actin organisation but enhanced proliferative

potential in comparison to the trailing edge. Therefore we consider this as collective invasion with growing edge as CD49f cells had an association with proliferation. Similar observations are reported in the case of morphogenesis in mammary tissue where cellular extensions or actin rich protrusions are absent in the front of advancing mammary ducts; but present in the luminal side [30]. Expression of CD49f at the leading edge increases as the phalloidin rich invasion protrusions become more prominent (Fig. 2E,F).This might suggest two points: 1) that cells switch to CD49f+ cells as they invade out and attach to the substrate. 2) as there is an increase in phalloidin stress fibers with increase in CD49f expression with growing cell protrusions; it's likely that CD49f expression at the leading edge might be essential for making the cells behind more migratory [15,32,33].

Our flow cytometry analysis of primary cancers was only partially revealing; in the absence of clinical outcome information from that particular cohort. It does however suggest a link with high CD66 expression and sarcomatoid cells and further validate the EMT profiles that we obtained by immunostaining as shown by association with TGFβ. Tumourigenic progression is clearly dependent on signals such as TGFβ and they have not been easy to resolve conclusively given the plethora of studies which have shown both a pro-oncogenic and tumour suppressive role for this pathway [22]. The data presented in this study is consistent with a link between CD66 and TGFβ expression. However, the cohort analysis based on broad expression patterns of CD66, CD49f and TGFβ reveal that the expression correlates that we see with metastatic samples (from different centers) does not necessarily lend itself to an easy explanation for local recurrences. Even well studied CD44+ve CD24−ve breast cancer stem cell subset has shown to be not associated with OS, but favouring distant metastasis [34]. It is likely that there might be different subsets of

CD66 with differentiation status or that there is heterogeneity based on isoforms. Also the significance of intra-cellular staining of CD66 observed in the retrospective study remains to be explored. In conclusion, while we provide a both mechanistic and clinical insight into the role of CD66 in human cervical cancer invasion and metastasis, this study emphasises that there might be diverse mechanisms of progression of local recurrences which are still poorly understood.

Conflict of interest statement

The authors have declared that no conflict of interest exists.

Acknowledgements

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejca.2016.03.072.

Ethical approval

All human patient samples studies are approved by institutional ethical committee of Kidwai Hospital and Cancer Institute (WIA). Experiments were done in compliance with ethical guidelines and without any clinical interventions.

References

[1] Marusyk A, Almendro V, Polyak K. Intra-tumour heterogeneity: a looking glass for cancer? Nat Rev Cancer 2012;12:323−34. http://dx.doi.org/10.1038/nrc3261.

[2] Blumenthal RD, Leon E, Hansen HJ, Goldenberg DM. Expression patterns of CEACAM5 and CEACAM6 in primary and metastatic cancers 2007;15:8809−17. http://dx.doi.org/10.1186/1471-2407-7-2.

[3] Johnson B. Emerging role and targeting of carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) in human malignancies n.d.;6:1−30.

[4] Lewis-Wambi JS, Cunliffe HE, Kim HR, Willis AL, Jordan VC. Overexpression of CEACAM6 promotes migration and invasion of oestrogen-deprived breast cancer cells. Eur J Cancer 2008;44: 1770−9. http://dx.doi.org/10.1016/j.ejca.2008.05.016.

[5] Gemei M, Mirabelli P, Di Noto R, Corbo C, Iaccarino A, Zamboli A, et al. CD66c is a novel marker for colorectal cancer stem cell isolation, and its silencing halts tumour growth in vivo. Cancer 2013;119:729−38. http://dx.doi.org/10.1002/cncr.27794.

[6] Bajaj J, Maliekal TT, Vivien E, Pattabiraman C, Srivastava S, Krishnamurthy H, et al. Notch signaling in CD66+ cells drives the progression of human cervical cancers. Cancer Res 2011;71: 4888−97. http://dx.doi.org/10.1158/0008-5472.CAN-11-0543.

[7] Pattabiraman C, Hong S, Gunasekharan VK, Pranatharthi A, Bajaj J, Srivastava S, et al. CD66+ cells in cervical precancers are partially differentiated progenitors with neoplastic traits. Cancer Res 2014;74:6682−92. http://dx.doi.org/10.1158/0008-5472.CAN-14-1032.

[8] Cheung KJ, Gabrielson E, Werb Z, Ewald AJ. Collective invasion in breast cancer requires a conserved basal epithelial program. Cell 2013;155:1639−51. http://dx.doi.org/10.1016/j.cell.2013.11.029.

[9] Roosmalen W Van, Dévédec SE Le, Golani O, Smid M, Pulyakhina I, Timmermans AM, et al. Tumour cell migration screen identifies SRPK1 as breast cancer metastasis determinant 2015;125:1−17. http://dx.doi.org/10.1172/JCI74440DS1.

[10] Westcott JM, Prechtl AM, Maine E a, Dang TT, Esparza M a, Sun H, et al. An epigenetically distinct breast cancer cell subpopulation promotes collective invasion 2015;125:1−17. http://dx.doi.org/10.1172/JCI77767.a.

[11] Goel HL, Gritsko T, Pursell B, Chang C, Shultz LD, Greiner DL, et al. Regulated splicing of the α6 integrin cytoplasmic domain determines the fate of breast cancer stem cells. Cell Rep 2014;7: 747−61. http://dx.doi.org/10.1016/j.celrep.2014.03.059.

[12] Lo P, Kanojia D, Liu X, Singh UP, Berger FG, Wang Q, et al. CD49f and CD61 identify Her2/neu-induced mammary tumour-initiating cells that are potentially derived from luminal progenitors and maintained by the integrin − TGF b signaling 2011: 1−13. http://dx.doi.org/10.1038/onc.2011.439.

[13] López J, Poitevin A, Mendoza-martínez V, Pérez-plasencia C, García-carrancá A. Cancer-initiating cells derived from established cervical cell lines exhibit stem-cell markers and increased radioresistance. BMC Cancer 2012;12:48. http://dx.doi.org/10.1186/1471-2407-12-48.

[14] Aplin JD, Dawson S, Seif MW. Abnormal expression of integrin oc6, B4 in cervical intraepithelial neoplasia 1996:240−5.

[15] Mercurio AM, Rabinovitz I, Shaw LM. The alpha 6 beta 4 integrin and epithelial cell migration. Curr Opin Cell Biol 2001;13: 541−5.

[16] Rabinovitz I, Toker A, Mercurio AM. Protein kinase C-dependent mobilization of the α6β4 integrin from hemidesmosomes and its association with actin-rich cell protrusions drive the chemotactic migration of carcinoma cells. J Cell Biol 1999;146:1147−59. http://dx.doi.org/10.1083/jcb.146.5.1147.

[17] King TE, Pawar SC, Majuta L, Sroka IC, Wynn D, Demetriou MC, et al. The role of alpha 6 integrin in prostate cancer migration and bone pain in a novel xenograft model. PLoS One 2008:3. http://dx.doi.org/10.1371/journal.pone.0003535.

[18] Reymond N, Im JH, Garg R, Vega FM, Borda d'Agua B, Riou P, et al. Cdc42 promotes transendothelial migration of cancer cells through β1 integrin. J Cell Biol 2012;199:653−68. http://dx.doi.org/10.1083/jcb.201205169.

[19] Mercurio a M, Rabinovitz I. Towards a mechanistic understanding of tumour invasion−lessons from the alpha6beta4 integrin. Semin Cancer Biol 2001;11:129−41. http://dx.doi.org/10.1006/scbi.2000.0364.

[20] Hegerfeldt Y, Tusch M, Bröcker E-B, Friedl P. Collective cell movement in primary melanoma explants. Cancer Res 2002;62: 2125−30.

[21] Yamaguchi N, Mizutani T, Kawabata K, Haga H. Leader cells regulate collective cell migration via Rac activation in the downstream signaling of integrin β1 and PI3K. Sci Rep 2015;5: 7656. http://dx.doi.org/10.1038/srep07656.

[22] Akhurst RJ, Derynck R. TGF-beta signaling in cancer − a double-edged sword. Trends Cell Biol 2001;11:S44−51. http://dx.doi.org/10.1016/S0962-8924(01)02130-4.

[23] Massagué J. TGFβ in Cancer. Cell 2008;134:215–30. http://dx.doi.org/10.1016/j.cell.2008.07.001.

[24] Ki K, Tong S, Huh C, Lee J, Lee S, Chi S. Expression and mutational analysis of TGF-β/Smads signaling in human cervical cancers 2009;20:117–21. http://dx.doi.org/10.3802/jgo.2009.20.2.

[25] Hazelbag S, Gorter A, Kenter GG, van den Broek L, Fleuren G. Transforming growth factor-beta1 induces tumour stroma and reduces tumour infiltrate in cervical cancer. Hum Pathol 2002;33:1193–9. http://dx.doi.org/10.1053/hupa.2002.130109.

[26] Chui MH. Insights into cancer metastasis from a clinicopathologic perspective: epithelial-mesenchymal transition is not a necessary step. Int J Cancer 2013;132:1487–95. http://dx.doi.org/10.1002/ijc.27745.

[27] Jaspers JE, Sol W, Kersbergen A, Schlicker A, Guyader C, Xu G, et al. BRCA2-deficient sarcomatoid mammary tumours exhibit multidrug resistance. Cancer Res 2015;75:732–41. http://dx.doi.org/10.1158/0008-5472.CAN-14-0839.

[28] Kalluri R, Weinberg RA. Review series The basics of epithelial-mesenchymal transition 2009;119. http://dx.doi.org/10.1172/JCI39104.1420.

[29] Friedl P, Hegerfeldt Y, Tusch M. Collective cell migration in morphogenesis and cancer 2004;449:441–9.

[30] Ewald AJ, Brenot A, Duong M, Chan BS, Werb Z. Collective epithelial migration and cell rearrangements drive mammary branching morphogenesis. Dev Cell 2008;14:570–81. http://dx.doi.org/10.1016/j.devcel.2008.03.003.

[31] Ilina O, Friedl P. Mechanisms of collective cell migration at a glance 2009:3209–13. http://dx.doi.org/10.1242/jcs.036525.

[32] Rabinovitz I, Toker A, Mercurio AM. Protein kinase C-dependent mobilization of the alpha6beta4 integrin from hemidesmosomes and its association with actin-rich cell protrusions drive the chemotactic migration of carcinoma cells. J Cell Biol 1999;146:1147–60.

[33] Cruz-Monserrate Z, O'Connor KL. Integrin alpha 6 beta 4 promotes migration, invasion through Tiam1 upregulation, and subsequent Rac activation. Neoplasia 2008;10:408–17. http://dx.doi.org/10.1593/neo.07868.

[34] Abraham BK, Fritz P, McClellan M, Hauptvogel P, Athelogou M, Brauch H. Prevalence of CD44+/CD24−/low cells in breast cancer may not be associated with clinical outcome but may favor distant metastasis. Clin Cancer Res 2005;11:1154–9.