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Expression of ALCAM in Clinical Colon Cancer and Relationship With Patients' Treatment Responses

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Abstract. *Background/Aim: Activated leukocyte cell adhesion molecule (ALCAM) plays an important role in cancer via its homotypical and heterotypical interactions with ALCAM or other proteins and can also mediate cell-cell interactions. The present study investigated the expression of ALCAM in relation to epithelial–to–mesenchymal transition (EMT) markers and its downstream signal proteins including Ezrin-Moesin-Radixin (ERM), in clinical colon cancer and in the progression of the disease. Materials and Methods: Expression of ALCAM was determined in a clinical colon cancer cohort and assessed against the clinical pathological factors and outcome, together with the expression patterns of the ERM family and EMT markers. ALCAM protein was detected using immunohistochemistry. Cell line models, with ALCAM knock-down and over-expression, were established and used to test cells' responses to drugs. Results: Tumours from patients who had distant metastasis and died of colon cancer had low levels of ALCAM. Dukes B and C tumours also had lower ALCAM expression than Dukes A tumours. Patients with high levels of ALCAM had a significantly longer overall and disease-free survival than those with lower ALCAM levels (p=0.040 and p=0.044). ALCAM is not only significantly correlated with SNAI1 and TWIST, also positively correlated with SNAI2. ALCAM enhanced the adhesiveness of colorectal cancer, an effect inhibited by both sALCAM and SRC inhibitors. Finally, high ALCAM expression rendered cells resistant, especially to 5-fluorouracil. Conclusion: Reduced expression of ALCAM in colon cancer is an indicator of disease progression and a poor prognostic indicator for patient's survival. However, ALCAM can enhance the adhesion ability of cancer cells and render them resistant to chemotherapy drugs.*

Activated leukocyte cell adhesion molecule (ALCAM), also known as CD166, plays a pivotal role in mediating cell adhesion, including in cancer cells. It has a rather ubiquitous distribution in the human body, and is and is highly expressed highly expressed in the nervous tissue, pancreas, thyroid, and parathyroid tissues. ALCAM confers homotypic and heterotypic adhesions between the same and different cell types *via* both homotypic and heterotypic protein interactions. Intracellularly, ALCAM anchors to the skeleton by interacting with the Ezrin-Moesin-Radixin (ERM) family of proteins (1, 2).

ALCAM has been studied for its role in cancer and cancer development, which remains an active topic. In bronchial epithelial cells, ALCAM promotes cell growth while it can inhibit the metastasis of lung cancer (1). In breast cancer, ALCAM has been shown to promote adhesion, proliferation, and tumour growth (2). In some other tumours such as glioma, higher ALCAM can induce migration (3). In clinical cancers, ALCAM has a rather diverse expression pattern and temporally opposite role in disease progression and correlation with prognosis (4). For example, in mesothelioma (5), gastric cancer (6), oesophageal cancer (7) and pancreatic cancer (8) high levels of ALCAM in tumours represent an indicator for poor prognosis. ALCAM has been recently found to promote distant metastasis and regional/transcoelomic spreading by promoting the seed and soil process (9, 10). In other cancer types however, ALCAM has been reported to be a favourable prognostic factor including breast cancer (11), thyroid cancer (12), prostate cancer (13), although localization of ALCAM in cancer cells, namely cytoplasmic and membranous compartments, in these tumour types, have shown a different connection with the disease progression.

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Key Words: ALCAM, CD166, clinical staging, clinical outcome, survival, drug response.

Table I. *Primers used in the study.*

Target	Forward primer	Reverse primer*
ALCAM	ttatcataccttgccgatt	gggtggaagtcatggtatag
ALCAM	caggaggttgaaggactaaa	actgaacctgaccgtacagggatcagttttctttgtca
SLUG (SNAI2)	ctccaaaaagccaaactaca	actgaacctgaccgtacagaggatctctggttgtggta
SNAI1	tctttcctcgtcaggaagc	actgaacctgaccgtacactgctggaaggtaaactctg
TWIST	aagctgagcaagattcagac	actgaacctgaccgtacagaggacctggtagaggaagt
CDH1 (E-cadherin)	caggagccagacacatttat	actgaacctgaccgtacagttcttcacgtgctcaaaat
CDH2 (N-cadherin)	caacgacgggttagtcac	actgaacctgaccgtacagctaatggcacttgattttc
EGFR (Her1)	gacctccatgcctttgagaa	actgaacctgaccgtacagcacaaatttttgtttcctga
Her2	gtggacctggatgacaag	actgaacctgaccgtacagaccacgaccagcagaat
Her3	ccccacaccaagtatcagta	actgaacctgaccgtacaacacaggatgtttgatccac
Her4	ctgctgagttttcaaggatg	actgaacctgaccgtacaaacttgctgtcatttggact
GAPDH	ggctgcttttaactctggta	gactgtggtcatgagtcctt
GAPDH	aaggtcatccatgacaactt	actgaacctgaccgtacagccatccacagtcttctg

*Sequence "actgaacctgaccgtaca" is the Z-sequence for QPCR reaction.

Table II. *Levels of ALCAM transcript expression in colon tissues.*

	Variable	N	ALCAM transcript [Median (Q1-Q3)]	*p*-Value
Tissue type	Normal	80	57 (7-1,641)	0.4695*
	Tumour	94	21 (2-3,144)	
Paired types	Paired normal	68	42 (3-1,571)	0.4398*
	Paired tumour	68	13 (1-1,459)	
Node status	Negative	39	14 (1-2,491)	0.9859*
	Positive	31	19 (1-3,144)	
TNM staging	TNM1	9	4,224 (271-12,586)	
	TNM2	30	7 (1-235)	0.0188*
	TNM3	26	5 (1-1,523)	0.033*
	TNM4	6	8,876 (505-97,640)	0.68*
Dukes staging	Dukes A	7	4,224 (23-7,217)	0.077$
	Dukes B	33	9 (1-434)	0.1092*
	Dukes C	32	22 (1-3,228)	0.2643*
	Dukes BC	65	14 (1-1,348)	0.1486*
Invasion	No Invasion	50	14 (2-640)	0.7885*
	Invasive	26	22 (1-3,365)	
Disease-free	Disease-free	35	36 (1-3,144)	0.0452*
	With colon cancer-related	23	4 (0-24)	

*Pairwise comparison using Mann-Whitney *U*-test. $Groupwise comparison using Kruskal-Wallis test.

Colon cancer is the third most common cancer worldwide and has a higher incidence in developed countries (14). In the USA, the 5-year survival is around 60%, while in less developed countries it is lower than 40%. The study of ALCAM in this cancer type remains controversial. In a tissue array based study with 299 patients, low levels of ALCAM protein staining were shown to be a favourable prognostic factor for overall survival of the colon cancer patients (7). ALCAM-negative colon tumour tends to have a greater risk of developing lymph node metastasis and distant metastasis than ALCAM-positive tumours (15). However, an early study has shown that positive membrane ALCAM, not cytoplasmic ALCAM, is an adverse prognostic factor (16), which is in clear contrast to another study, which reported that cytoplasmic ALCAM was associated with a poor clinical outcome of patients (17). ALCAM has been shown to be a potential biomarker of epithelial–to–mesenchymal transition (EMT) (18). As for the distant metastasis of cancer, ALCAM is positively correlated with liver metastasis of colorectal cancer by interacting with SOSTDC1 (19). For clinical therapy, it has also been found that when colon cancer patients are treated with 5-fluorouracil, ALCAM expression

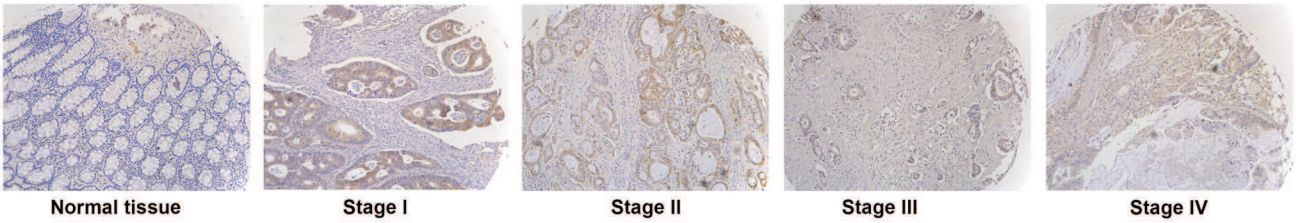

Figure 1. *Immunohistochemical (IHC) staining for ALCAM in normal colon tissues and colon cancer tissue with different stages. IHC staining was carried out using CO2161b tissue microarray.*

Table III. *ALCAM staining in normal and colon adenocarcinoma tissues in CO2161b tissue microarray.*

Tissue	IHC stain score 0,1	IHC stain score 2,3
Normal tissue	8 (100%)	0
Adenocarcinoma	119 (68%)	56 (32%)

IHC: Immunohistochemistry score.

Table IV. *ALCAM and patient's survival.*

Clinical outcome	Hazard ratio (95%CI)	*p*-Value*
Overall survival	0.416 (0.175-0.988)	0.047
Disease-free survival	0.423 (0.178-1.006)	0.05
Distant metastasis-free survival	0.484 (0.181-1.269)	0.149

*By Cox regression. CI: Confidence interval.

Table V. *ALCAM transcript expression and patient's response to chemotherapies.*

Drug	Response	Expression in response groups			AUC (*p*-Value by ROC)
		n	Median (min-max)	*p*-Value (Mann-Whitney)	
Bevacizumab	Responder	28	484 (217-1,525)	0.46	0.559 (0.23)
	Non-responder	26	605 (203-1,623)		
5-FU	Responder	148	487 (8-2,457)	0.37	0.530 (0.18)
	Non-responder	155	458 (38-1,773)		
Irinotecan	Responder	60	552 (8-2,068)	0.39	0.544 (0.19)
	Non-responder	69	503 (38-1,623)		
Oxaliplatin	Responder	97	454 (93-2,457)	0.36	0.540 (0.18)
	Non-responder	77	430 (127-1,773)		
Capecitabine	Responder	16	312 (184-1,145)	0.96	0.505 (0.48)
	Non-responder	42	333 (119-870)		

AUC: Area under the curve; ROC: receiver operating characteristic.

is not an important factor, which can help to predict the response (20). In contrast, Sim *et al*. reported that patients with high ALCAM had a longer disease-free survival when treated with 5-fluorouracil (5-FU) (21). Thus, the impact of ALCAM remains unclear in colon cancer,

The present study investigated the expression of ALCAM at transcript and protein levels, in a cohort of colorectal cancer and, by creating cell models with differential expression of ALCAM, tested cells' response to chemotherapeutic drugs.

Materials and Methods

Colorectal cohort for gene transcript analysis. A cohort of 94 colorectal fresh tumour tissues and matched normal tissues (15 cm away from tumour margins), were collected immediately after surgery at the University Hospital of Wales (Heath Park, Cardiff, UK), as we previously reported (22, 23). Patients with other cancers, with family history of cancers and patients who received chemotherapy before surgery, were excluded from the study. The median age was 73 years (range=25-88 years). The collection was made under research ethics approval by the local research ethics committee, Bro Taf Research Ethics Committee (Ref. 05/DMD/3562). The clinical, pathological and outcome information were retrospectively collected after surgery and during the follow-up. Tissues, stored at –80˚C, were processed using frozen sections for RNA extraction (Sigma-Aldrich; Merck KGaA, Dorset, UK) and reverse transcription using a reverse transcription kit from Promega Corporation (Southampton, UK).

Colon cancer cell lines. Human colorectal cancer cell lines, HT115, HRT18 and RKO were purchased from ECACC (European

Collection of Animal Cell Culture, Salisbury, UK). RKO cells were routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FCS), penicillin (100 unit/ml) and streptomycin (100 mg/ml).

Key materials. The plasmids which contained shRNA targeting ALCAM, a plasmid with expression cassette of human ALCAM, and control plasmids containing scramble sequence, were purchased from VectorBuilder (Chicago, IL, USA) and have been previously reported (4, 24). An ALCAM antagonist, soluble ALCAM, was purchased from R&D systems (Abingdon, UK). The fluorescence dye, DiI (1,1'-Dioctadecyl-3,3,3',3'Tetramethylindocarbocyanine Perchlorate), was purchased from Sigma-Aldrich (Dorset, UK). Small inhibitor to SRC, AZM475271, was from Tocris (Bristol, UK). Antibody to human ALCAM was from Novacastra (Milton Keynes, UK) and GAPDH was from Santa Cruz Biotechnologies (Santa Cruz, TX, USA).

Generation of genetically modified colon cancer cells. Colon cancer cells were transfected with the shRNA plasmids in order to establish ALCAM knock-down cell models. Cells were subject to selection with 2 μg/ml puromycin (Fisher Scientific, Oxford, UK) and, once tested for the success of genetic modification, were routinely maintained in a maintenance medium (with 0.2 μg/ml puromycin).

Quantitative analyses of gene transcripts. ALCAM transcripts, in cells and tissues, were determined using reverse transcription-quantitative polymerase chain reaction (RT-PCR) or quantitative RT-PCR, as previously reported (4, 25, 26). The levels of ALCAM in cells and tissues were determined by qPCR, with application of a molecular beacon based AmplifluorTM Uniprimer™ Universal qPCR system (Intergen Inc., Oxford, UK). The system was characterised by integrating a Z sequence (5'-ACTGAACCTGACCGTACA-3') to the FAM-tagged Uniprimer™ probe (Table I). The reaction and detection were carried out using a StepOnePlusTM Real-Time PCR System (Fisher Scientific). The amplification and detection conditions were: 95˚C for 10 min, 80 cycles of 95˚C for 10 s, 55˚C for 35 s (programmed for signal detection) and 72˚C for 10 s. The transcripts were quantified alongside an internal standard to allow calculation of relative transcript copy numbers.

Protein preparation and electrophoresis. Protein was extracted from cells with a lysis buffer containing NP40 and protein concentration quantified using a BioRad protein quantitation kit (Bio-Rad Laboratories, Hertfordshire, UK). Equal amounts of protein were loaded to an 8% SDS-PAGE gel and subject to electrophoresis. Protein was subsequently blotted onto PVDF membrane (Merck Millipore, Hertfordshire, UK) using a semi-dry blotter. The membrane was subject to blocking (containing 10% milk), probing with the primary (overnight at 4˚C) and secondary HRP conjugated antibody, each separated by extensive washing. Protein band was visualized after immersing the membrane in an EZ-ECL solution (equal parts of solution A mixed with solution B) (Geneflow Ltd., Litchfield, UK), on a G-BOX imager (Syngene, Cambridge, UK).

Immunohistochemical (IHC) analysis. The IHC staining for ALCAM was performed using a tissue microarray (TMA, CO2161b) (178 cases of adenocarcinoma, 26 cases of Mucinous adenocarcinoma, 2 signet-ring cell carcinoma and 8 normal colon tissue). Sections were dewaxed in xylene and rehydrated through a

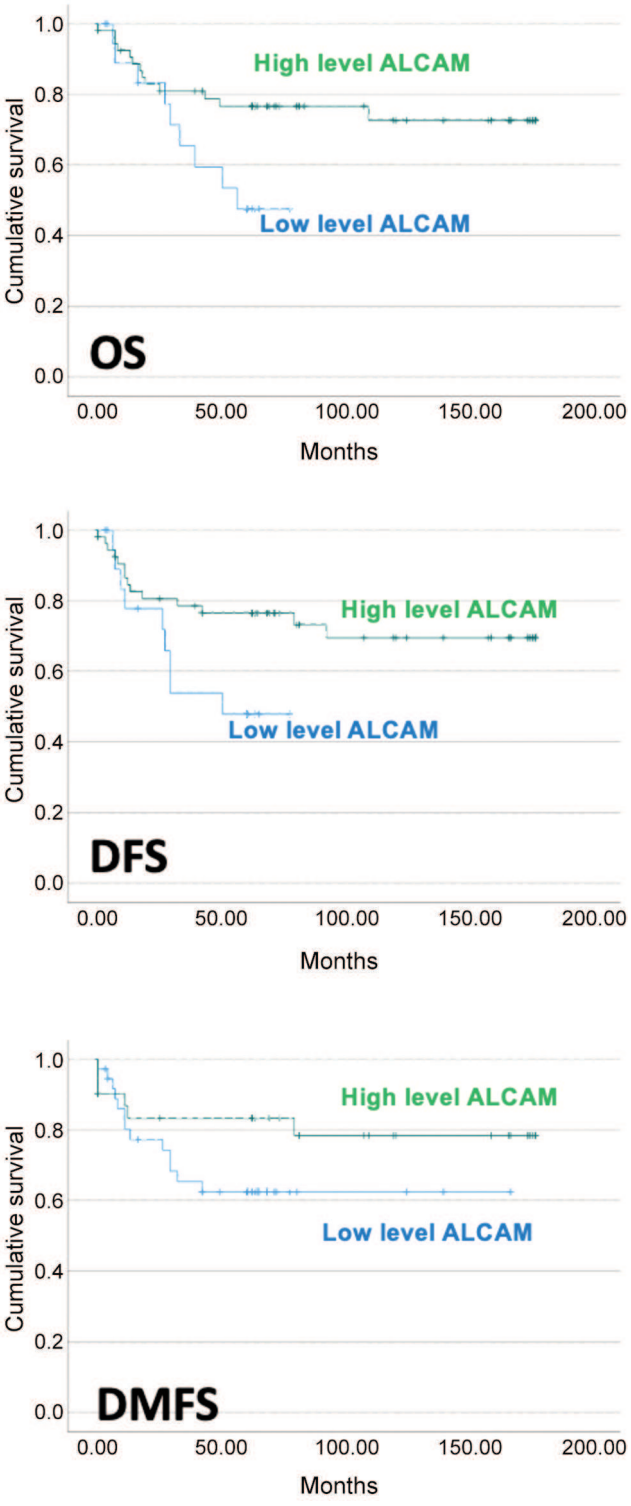

Figure 2. *ALCAM transcript expression and the clinical outcome of the patients. Shown are the overall survival (OS) (p=0.038), disease-free survival (DFS) (p=0.044), distant metastasis-free survival (DMFS) (p=0.137) in patients with different ALCAM expression. Cutoff value was based on the optimal receiver operating characteristic (ROC) curve cutoff value by ROC analysis.*

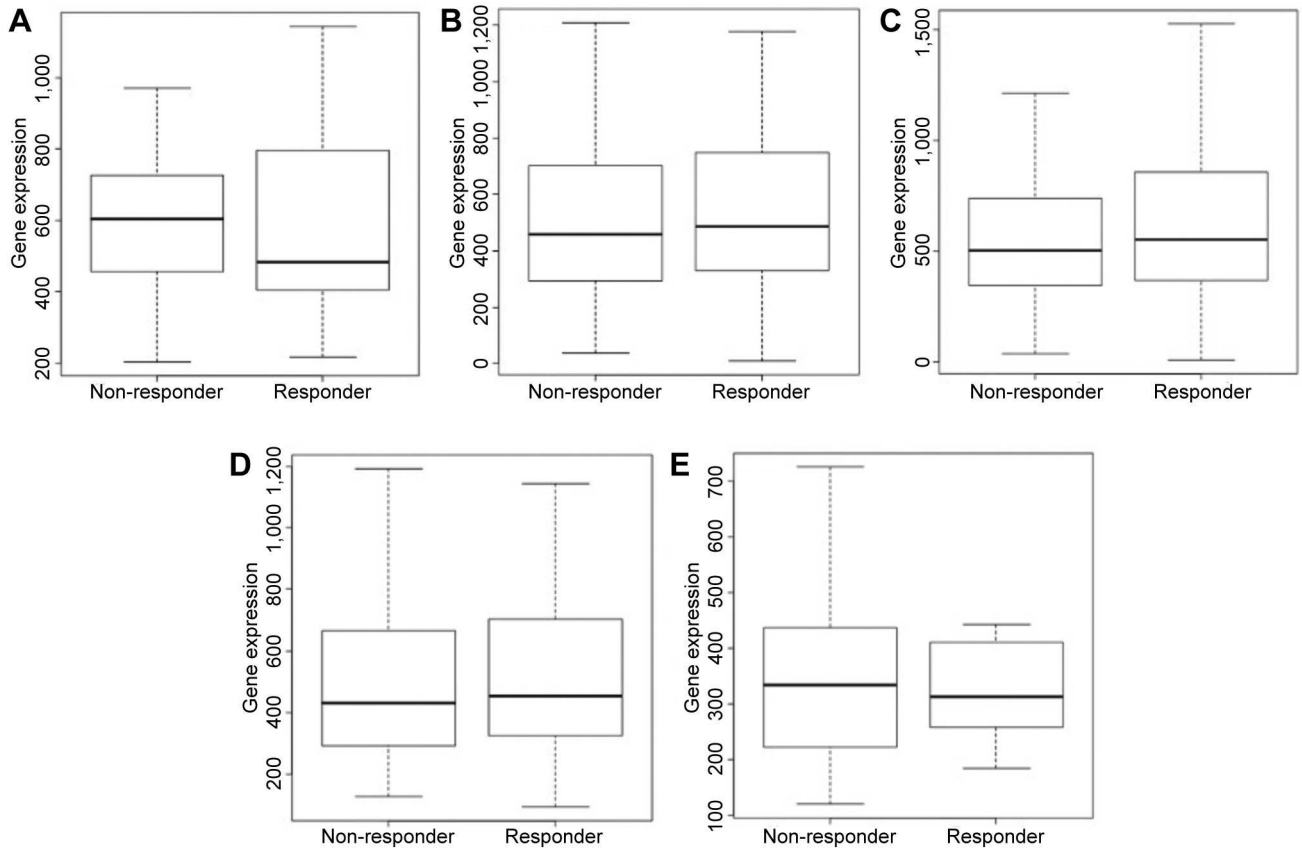

Figure 3. *ALCAM expression in patients who had or did not have a response to the chemotherapy drugs, including, A) Bevacizumab (Avastin); B) 5-fluorouracil; C) Irinotecan; D) Oxaliplatin; E) Capecitabine.*

graded series of ethanol/distilled water, ending with a final wash in PBS. Following a 2-hour blocking step with 10% horse serum (Sigma-Aldrich; Merck KGaA), the sections were incubated overnight at 4˚C with the appropriate primary antibody (diluted to a final concentration of 2 mg/ml in the blocking serum). After washing thoroughly in PBS, the staining protocol proceeded using the Vectastain Universal Elite ABC Kit (cat no. PK-6200; Vectastain Universal Elite ABC kit, Vector Laboratories, Newark, CA, USA) following the manufacturer's protocol. Briefly, sections were incubated for 30 min with the biotinylated secondary antibody from the kit, washed with PBS, incubated at room temperature for 30 min with ABC tertiary reagent before the staining was developed using 3,3'-Diaminobenzidine (DAB) substrate. The slides were then briefly washed using tap water prior to counterstaining with Gill's haematoxylin. This was followed by washing with tap water, dehydrating in a graded series of ethanol, clearing in xylene, and mounting with dibutylphthalate polystyrene xylene. The staining was examined using a light microscope and scored (0=negative, 1=weak, 2=moderate, 3=strong).

Implication of ALCAM in responses of patients to therapies and angiogenesis. The public dataset from The Cancer Genome Atlas (TCGA) was explored (27). The relationship of levels of ALCAM with patients' responses to chemotherapy was analysed using www.rocplot.com (accessed June 2022) (28). The responses were tested using the receiver operating characteristic (ROC) method for the chosen gene probes (201951_at), to allow classification of the patients based on their responses to chemotherapy. The levels of ALCAM in the chemo-responsive and non-responsive groups were compared using Mann-Whitney *U*-test.

Evaluation of cell adhesion and migration by electric cell-substrate impedance sensing (ECIS). We employed an established method, ECIS, to assess cell adhesiveness and migration of colon cancer cells. This was based on the ECIS Z-Theta model (Applied Biophysics, Troy, NY, USA) using a method previously described (4, 29, 30). Briefly, the 96W1E array was first prepared by clearing the gold surface with the built in function of ECIS Z-Theta. Colon cancer cells, control or ALCAM genetically modified cells, were added to the arrays in the presence or absence of soluble ALCAM (sALCAM) or SRC inhibitor. The arrays were monitored immediately for up to 20 hours.

Cell growth and cytotoxicity assays. Two days after transient transfection, cells were harvested and seeded in each well (5,000 cells in 100 μl medium) on a 96-well-plate, treated with indicated chemotherapy drugs at different concentrations. Serially diluted chemotherapy drugs including 5-FU (range=0.064-1,000 μM),

Table VI. *Correlation between ALCAM and epithelial–to–mesenchymal transition markers.*

Colon cancer	TWIST1	SLUG (SNAI2)	SNAI1	ECAD	NCAD
Correlation coefficient§	0.268**	0.293**	0.481**	0.214	–0.05
Sig. (2-tailed)	0.009	0.004	0	0.098	0.659
Normal colon	TWIST1	SLUG (SNAI2)	SNAI1	ECAD	NCAD
Correlation coefficient§	0.141	0.128	0.288*	–0.328*	–0.136
Sig. (2-tailed)	0.212	0.258	0.017	0.026	0.378

§Using Spearman ranked correlation test. *p<0.05, **p<0.01.

Table VII. *Correlation between ALCAM and the Her family members.*

Colon cancer	Her1	Her2	Her3	Her4
Correlation coefficient§	0.250*	–0.08	–0.147	0.207*
Sig. (2-tailed)	0.017	0.448	0.208	0.045
Normal colon	Her1	Her2	Her3	Her4
Correlation coefficient§	0.286*	0.402**	0.306**	–0.092
Sig. (2-tailed)	0.001	0	0.009	0.419

§Using Spearman ranked correlation test. *p<0.05, **p<0.01.

Table VIII. *Correlation between ALCAM and the Ezrin-Moesin-Radixin family members.*

Colon cancer	Ezrin	Moesin	Radixin	EHM2
Correlation coefficient§	0.118	–0.063	0.11	0.106
Sig. (2-tailed)	0.408	0.666	0.448	0.307
Normal colon	Ezrin	Moesin	Radixin	EHM2
Correlation coefficient§	0.179	–0.001	0.015	0.353**
Sig. (2-tailed)	0.263	0.994	0.926	0.001

§Using Spearman ranked correlation test. **p<0.01.

Docetaxel (DTX; range=0.064-1,000 nM), AG825 (range=0.032-500 μM) and Oxaliplatin (range=0.064-1,000 μM) were added into the 96-well plates. After incubation with chemotherapy drugs for 48 h, the cells were fixed with 4% formaldehyde at room temperature for 15 min, followed by crystal violet (0.5%) staining for 10 min. After gently washing the plate to remove the excess crystal violet, 100 μl of acetic acid (10%) was added into each well of the dry plate. Absorbance at the wavelength of 595 nm was read to assess the cytotoxicity of the drugs in each group. Each group was repeated three times. IC_{50} values were calculated based on logarithmic trend line.

Statistical methods. All statistical analyses were conducted using SPSS (version 27.0; IBM, Portsmouth, UK). Survival analysis was performed using the Kaplan-Meier with log ranked method and Cox Regression. Kruskal-Wallis test was used to check whether samples originate from the same distribution. Correlation was determined using Spearman's correlation methods. Pairwise sample comparisons were obtained using unpaired Student's *t*-test and Mann-Whitney *U*-test for normally and non-normally distributed data sets as appropriate. Comparison of multiple groups were conducted using ANOVA test followed by Bonferroni correction. p<0.05 was considered to indicate a statistically significant difference.

Results

Expression of ALCAM in colon tissues. In the Cardiff clinical cohort, qPCR results showed that there was no significant difference in the levels of the ALCAM transcript between

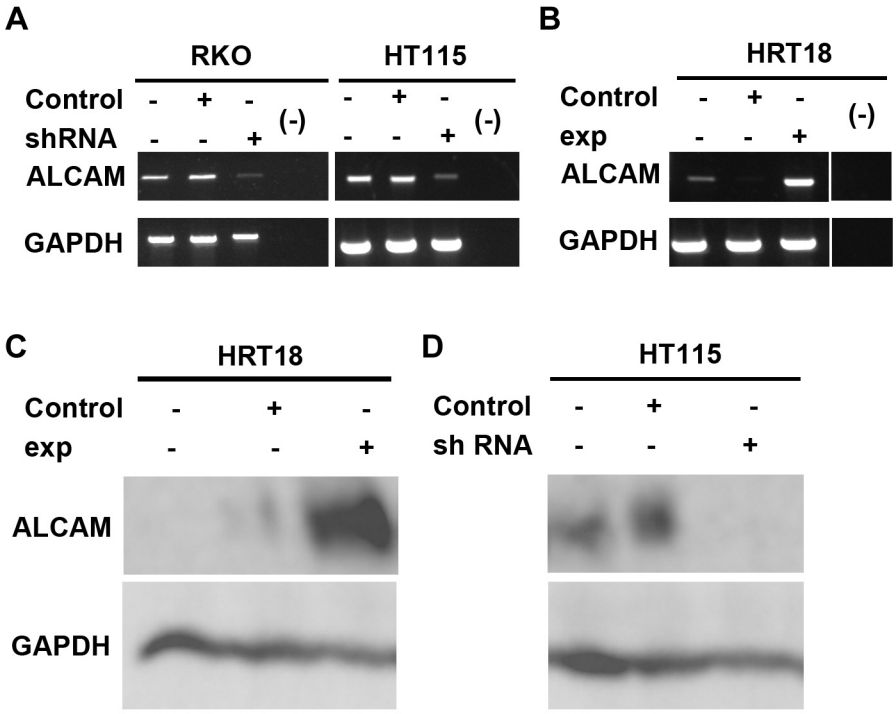

Figure 4. *Establishment of cell models with differential ALCAM expression. RKO and HT115 cell lines were transfected by scramble control (sc) and ALCAM shRNA plasmids to establish control group and ALCAM knockdown cell lines. For ALCAM over-expression, HRT18 cell line was transfected by ALCAM over-expression (exp) plasmids, and blank stuffer plasmids (stuffer) was applied to establish the control cell line. (A) The ALCAM transcripts level in RKO and HT115 wild type cell lines, control cell lines* $RKO^{sc\ control}$, $HT115^{sc\ control}$ *and ALCAM knock-down cell lines* $RKO^{ALCAM\ shRNA}$, $HT115^{ALCAM\ shRNA}$. *(B) ALCAM expression level in HRT18 wild type cell line, control cell line* $HRT18^{stuffer\ control}$ *and ALCAM over-expression cell line* $HRT18^{ALCAM\ exp}$. *(C) Western blot shows the ALCAM protein level in HRT18 wild type control,* $HRT18^{stuffer\ control}$ *and* $HRT18^{ALCAM\ exp}$ *cell line. (D) ALCAM protein level in HT115 wild type control,* $HT115^{sc\ control}$ *and* $HT115^{ALCAM\ shRNA}$ *cell line.*

normal and tumour tissues. ALCAM was down-regulated in TNM2 and TNM3 tumours compared with TNM1. Compared with tumours from the disease-free group, ALCAM in tumours from a relapsed colon cancer had a lower expression (Table II).

To further verify the ALCAM expression in colon cancer patients, a colon TMA was used for IHC staining. ALCAM expression was increased in colon cancer tissue compared with normal tissue ($p<0.001$, by Chi-square test) (Figure 1, Table III). There was a marginal increase of the staining from stage I to IV. This difference was not significant.

ALCAM and patients' clinical outcome. There was a significant difference in the overall survival; patients with high levels of ALCAM had a significantly longer overall survival (OS) than those with lower ALCAM (137.6±9.5 months *versus* 53.2±6.3 months, respectively, $p=0.040$) (Figure 2). Likewise, patients with higher ALCAM had a significantly longer disease-free survival (DFS) than those with lower ALCAM (132.6±10.1 months *versus* 49.3±7.0 months, respectively, $p=0.044$) (Figure 2). A similar trend was observed with distant metastasis-free survival (DMFS), although the difference did not reach significance ($p=0.117$). For both OS and DFS, ALCAM is a favourable prognostic indicator with hazard ratio at 0.416 for OS and 0.423 for DFS, again ALCAM does not appear to be a significant indicator for DMFS (HR=0.484, $p>0.05$) (Table IV).

Patients' response to chemotherapy and ALCAM expression. Previous studies reported that ALCAM is associated with EMT progression and affects the response to chemotherapy, which was also examined in the Cardiff clinical cohort. The results show that ALCAM has little effect on the response to different drugs used in clinical therapy, as demonstrated from the public dataset (Figure 3, Table V).

Correlation of ALCAM, EMT markers, ERM family and the Her family members. We analysed the correlation between ALCAM and a set of markers, including EMT markers (Table VI), the Her family (Table VII) and the ERM family (Table VIII), in normal colon and tumour tissues. In tumour tissues, ALCAM was significantly correlated with TWIST1, SNAI1

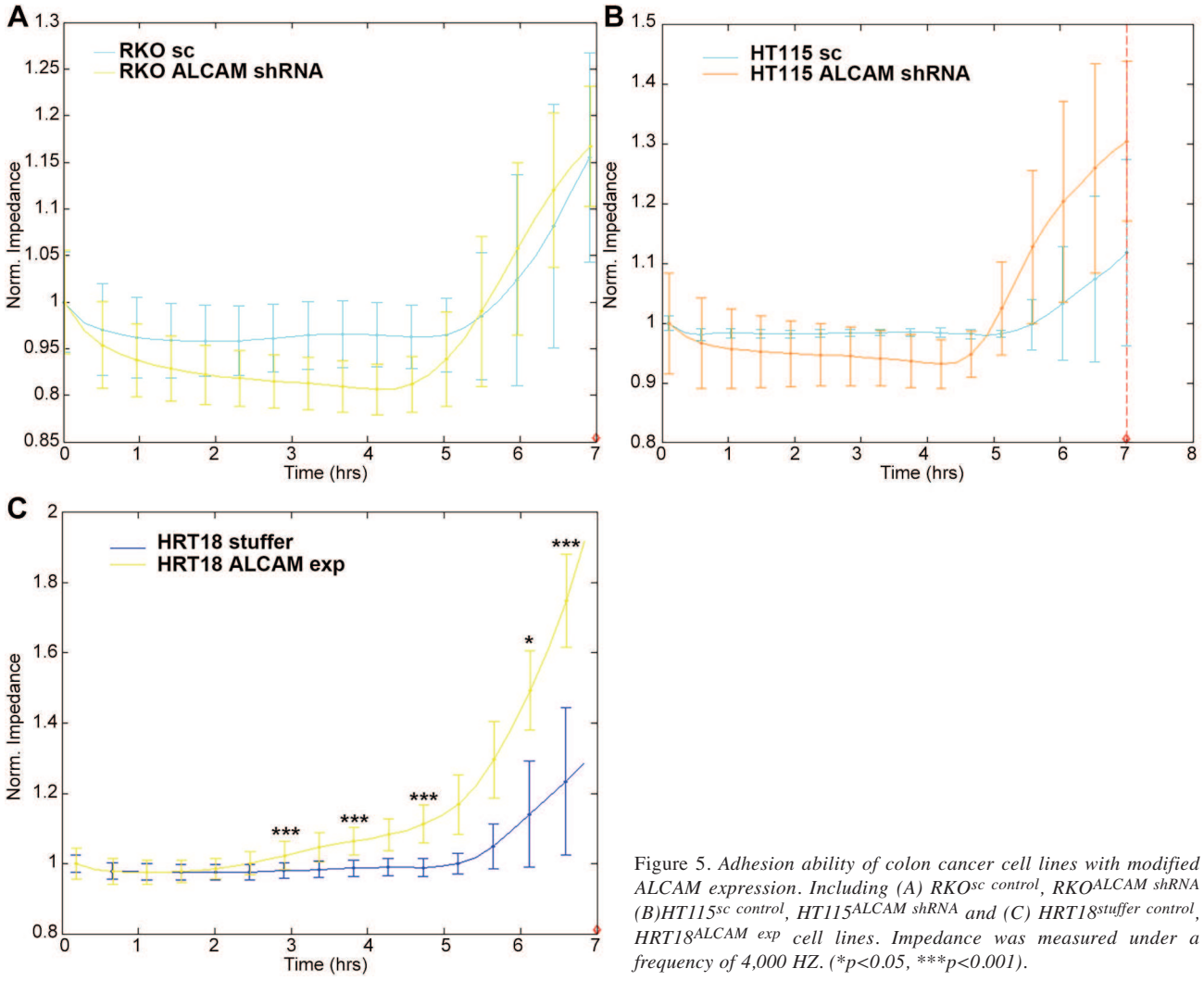

Figure 5. *Adhesion ability of colon cancer cell lines with modified ALCAM expression. Including (A) RKO$^{sc\ control}$, RKO$^{ALCAM\ shRNA}$ (B)HT115$^{sc\ control}$, HT115$^{ALCAM\ shRNA}$ and (C) HRT18$^{stuffer\ control}$, HRT18$^{ALCAM\ exp}$ cell lines. Impedance was measured under a frequency of 4,000 HZ. (*p<0.05, ***p<0.001).*

and SNAI2. In contrast, ALCAM was negatively correlated with E-cadherin in normal tissue but not in tumour tissues (Table VI). In normal tissues, ALCAM had significant correlation with Her1, Her2, and Her3, whereas in tumour tissues this was significant with Her1 and Her4 (Table VII). Finally, the relationship between ALCAM and the ERM family members, the cytoskeletal anchoring proteins for ALCAM in the cells, was also analysed. The only significant correlation was observed between ALCAM and EHM2 in normal tissues, but not in tumour tissues (Table VIII).

Generation of ALCAM knock-down and over-expression cell models. Three human colon cancer cell lines, with differing expression profiles of ALCAM, were chosen to create cell models. These were RKO and HT115 cells, that expressed high levels of ALCAM, and HRT18 cells, which expressed low levels of ALCAM (Figure 4). ALCAM knock-down, by way of shRNA, was successfully achieved in RKO and HT115 cells (Figure 4 left). An ALCAM over-expression model was also established in HRT18 cells following transfection with an ALCAM expression plasmid (Figure 4, right).

ALCAM expression and cell function. ALCAM was positively correlated with EMT progression, which can affect cell adhesion and migration. ECIS was used to investigate whether ALCAM expression could affect the cell adhesion ability. After ALCAM knock-down in RKO and HT115 cell lines, there was no significant change (Figure 5A and B). In the HRT18 cell line with ALCAM over-expression, the adhesion ability was significantly higher compared with the control group transfected by blank stuffer plasmid (Figure 5C). Furthermore, a cell growth assay showed that ALCAM did not affect proliferation in colon cancer cells (Figure 6).

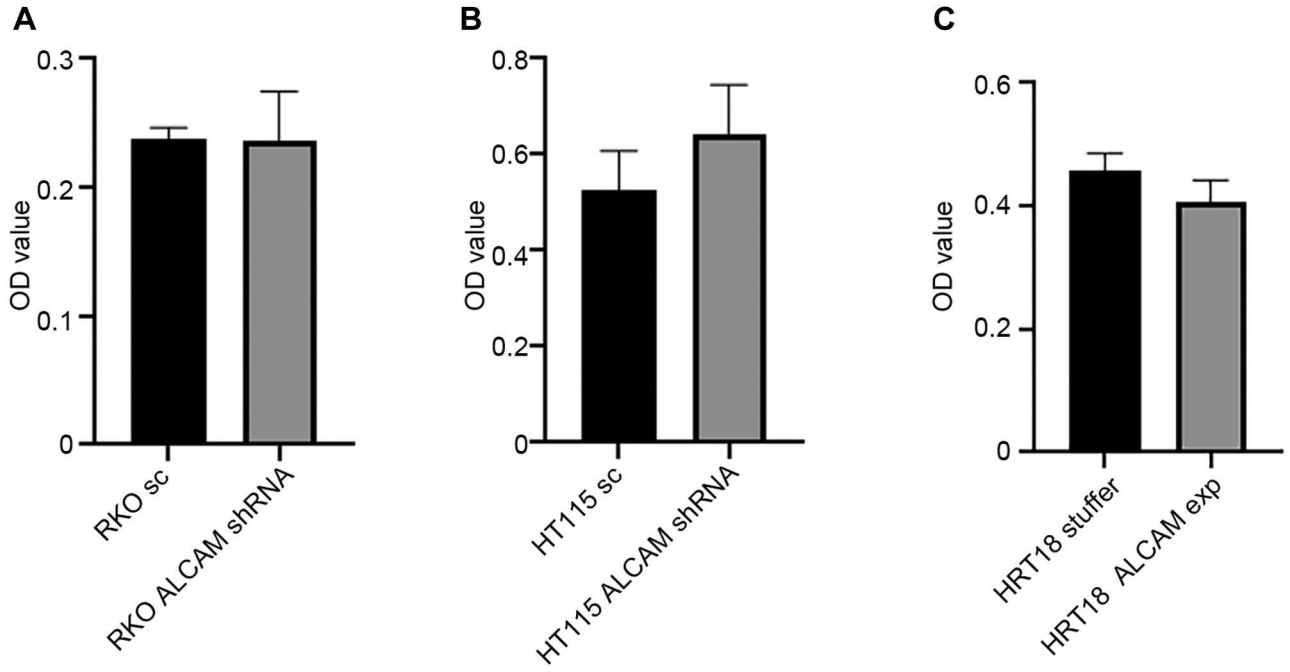

Figure 6. *ALCAM and the proliferation of colon cancer cells. For each cell line, 5,000 cells were seeded into 96-well plates. Following a 3-days' incubation, the optical density (OD) value was measured. Cell lines with modified ALCAM expression are shown including RKO (A), HT115 (B), and HRT18 (C).*

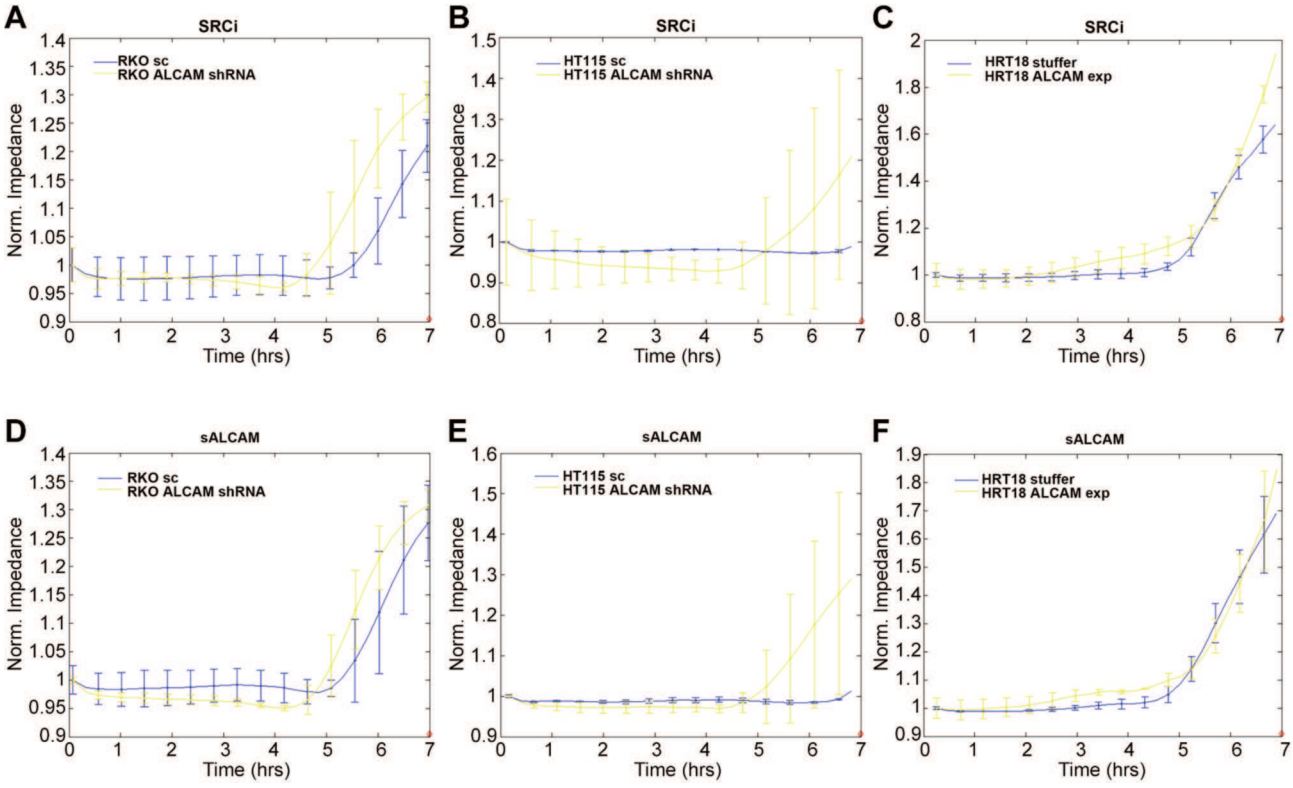

Figure 7. *SRCi and sALCAM can affect adhesion. (A) RKO, (B) HT115 and (C) HRT18 cell lines with modified ALCAM were treated with 400 nM SRC inhibitor (AZM475271). 2.5 μg/ml sALCAM was used to treat the (D) RKO (E) HT115 and (F) HRT18 with modified ALCAM expression. ECIS was applied to measure the adherence ability.*

Table IX. *IC_{50} of chemotherapy drugs in colon cancer cell lines with modified ALCAM expression.*

Cell lines	5FU (μM)	Docetaxel (nM)	Oxaliplatin (μM)
RKO-SC	10.936	4.7325	1.6805
RKO-shALCAM	4.7686	2.2134	1.938
HTT15-SC	3.4265	11.422	4.8022
HT115-shALCAM	1.5874	5.0569	4.7751
HRT18-Stuffer Control	6.8696	99.363	6.6408
HRT18-ALCAMexp	15.487	88.214	18.811

Table X. *IC_{50} of Her2 inhibitor in colon cancer cell lines with modified ALCAM expression.*

Cell lines	AG825 (μM)
RKO-SC	26.376
RKO-shALCAM	22.288
HT115-SC	8.999
HT115-shALCAM	9.8998
HRT18-Stuffer control	34.224
HRT18-ALCAMexp	35.717

Compared with cells incubated in control medium, RKO, HT115 and HRT18 with higher ALCAM expression, treated with 400 nM SRC inhibitor (AZM475271), had reduced adhesion ability compared with these three cell lines with a lower ALCAM expression (Figure 5 and Figure 7A, B, and C). A soluble ALCAM was also found to have a similar effect to SRC inhibitor, which inhibited the adhesion ability of colon cancer cells (Figure 5 and Figure 7D, E, and F).

ALCAM expression and cells responses to chemotherapeutic drugs. Using the cell models generated here, we further validated responses of cell lines to chemotherapy drugs (Table IX and Table X). Lower ALCAM expression was associated with a lower IC_{50} of 5-FU and docetaxel, in all three different cell lines. For oxaliplatin, this correlation was only observed in the HRT18 cell line. Furthermore, we also examined the effect of Her2 inhibitor (Table X). The response to AG825 did not appear to correspond to the levels of ALCAM in the cells (Table X).

Discussion

In the present study, we reported that ALCAM transcript expression level is a favourable prognostic marker in colon cancer. High levels of ALCAM were observed in patients with longer overall, disease-free, and metastasis-free survival.

The clinical association between ALCAM and colon cancer has remained unclear. Previously, it was shown that ALCAM expression, examined using immunohistochemical staining, was a favourable factor for the patients (13) and that ALCAM negative tumours had a high tendency for lymph node metastasis (15). Our study adds further evidence to that of Tachezy's, that in addition to protein level, ALCAM transcript level is also a good prognostic indicator. Although we did not detect a significant difference of ALCAM transcript levels between node negative and node positive tumours, we have shown a significant reduction of ALCAM transcript in TNM2 and TNM3 tumours. In consideration of the sharp contrast when assessing the subcellular location of ALCAM and the clinical significance (16, 17), our present study, together with that of Tachezy, indicates that assessment of total transcript and total protein in tumour tissues may be a more practical way of evaluation of clinical correlations in colon cancer.

There has been some evidence that patients with higher ALCAM may have a better outcome, after treatment with 5-FU (21). Here, we interrogate a public database which has some limited information on the response of patients to drug treatment and the ALCAM transcript levels. It was surprising that there was no significant correlation between the levels of ALCAM and patients' response to chemotherapeutic drugs including 5-FU, irinotecan, oxaliplatin and capecitabine, but not to Avastin. Our cell models do, however, show that low levels of ALCAM rendered them more sensitive to 5-FU, and HT115 and RKO cells to docetaxel but had little effect on their response to oxaliplatin. Collectively, this suggests that ALCAM may indeed have some impact, at the cellular level, on cells' response to drugs. This clearly needs to be validated in clinical studies with a larger cohort size.

ALCAM expression can be triggered by increased TWIST expression in some colon cancer cell lines (18), tentatively indicating that ALCAM may be involved in the EMT process. Here, we showed that ALCAM is positively correlated with some EMT markers including SnaI1, Slug, and Twist in colon cancer tissues, yet negatively correlated with E-cadherin in normal colon tissues. This contrasting correlation in tumour and normal tissues is very interesting and may indicate, that at the tissue level, reduced ALCAM, and potentially in correlation with the EMT process, may contribute to the less aggressive tumour type, hence renders patients with a favourable clinical outcome. In the correlation analysis with the ALCAM anchorage proteins, there was no significant correlation with ezrin, moesin, and radixin. However, our ECIS-based cell analyses did not show a marked change in the adhesiveness of HT115 and RKO cells. HRT18 cells with over-expressed ALCAM did show a significant effect on adhesiveness and this was ameliorated by treating with an SRC kinase inhibitor. Although this result should not be over-interpreted, it does suggest a potential connection between ALCAM and the EMT process in this cancer type, which is certainly worth further exploration in the future.

There have been reports that ALCAM, when integrated with the expression of Her family members, namely EGFR/Her1 and Her2, may bear some clinical significance in assessing the prognosis and in determining cell behaviour, in this case in breast cancer and squamous cell carcinoma (31, 32). Here, we also examined the correlation between ALCAM and all four Her family members. Normal and tumour tissues showed a different pattern of correlations, in that ALCAM correlated with Her1 and Her4 in tumours and with Her1, Her2 and Her3 in normal tissues. This interesting finding may suggest that in the context of ALCAM in colon cancer, Her2 may not be a key player in cell's responses to drugs in the context of ALCAM expression, as it was shown that the Her2 inhibitor in the cell model did not respond differently in cells with a different ALCAM expression pattern.

In conclusion, higher ALCAM transcript levels in colon cancer tissues are a good prognostic indicator for patients and is, to some degree, inversely correlated with disease progression. ALCAM may also have a role in the drug response of colon cancer cells.

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Conflicts of Interest

The Authors declare no conflicts of interest in relation to this study.

Authors' Contributions

Conception: W.G.J; Experimentation: A.F., J.Z., Y.Y., F.R.; Histology: F.R., Z.F.; Formal Analysis: Z.F., J.Z., F.R., Y.Y., J.L.; Writing: A.F., J.Z., F.R., J.L, Y.Y; Resource: R.H., W.G.J. Writing and editing: Z.F., J.Z., F.R., J.L., R.H., W.G.J.; Funding: W.G.J.

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