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ALCAM, Activated Leukocyte Cell Adhesion Molecule, in Clinical Gastric Cancer and Patient's Response to Chemotherapies

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Abstract. *Background/Aim: Activated leukocyte cell adhesion molecule (ALCAM/CD166), a member of the immunoglobulin superfamily, has been shown to regulate cell adhesion through both homotypic and heterotypic interactions. In cancer, it might be involved in disease progression and chemotherapy drug resistance. The present study explored the clinical and prognostic significance of ALCAM in gastric cancer and its impact on patient's responses to neoadjuvant chemotherapies and cancer cells' response to chemodrugs in vitro. Materials and Methods: Two independent cohorts were included to evaluate the link between ALCAM and the clinical outcomes and pathological factors of the patients. The gastric cancer cell lines HGC27 and AGS were used to generate ALCAM knockdown cell models. The cytotoxicity of chemotherapy drugs was examined using ALCAM knockdown cell models. Results: Patients with gastric cancer who had high levels of ALCAM transcripts showed a significantly shorter overall survival in both cohorts (p=0.043 and 0.006, respectively). Patients who resisted to neoadjuvant chemotherapy had marginally higher levels of ALCAM than those responded (p=0.056). Patients with low levels of ALCAM expression and resisted to neoadjuvant chemotherapy had the worst clinical outcome with a significantly shorter overall survival (p=0.004) and disease-free survival (p=0.006), whereas such results did not appear in high ALCAM expression patients. ALCAM knockdown cells were more sensitive to Cisplatin, Oxaliplatin and 5-Fluorouracil compared with their respective control cells. Conclusion: ALCAM acts as a negative prognostic indicator in patients with gastric cancer and high levels of ALCAM expression result in increased chemotherapy drug resistance.*

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Key Words: ALCAM, CD166, gastric cancer, neoadjuvant chemotherapy, survival, drug resistance, gastric cell model.

Gastric cancer (GC) is one of the most common malignant tumours worldwide. It accounted for approximately 5.6% new cancer cases and 7.7% cancer-related death in 2020 according to the GLOBOCAN report (1). Despite recent advances in gastric cancer treatment, the long-term survival of patients with gastric cancer remains unsatisfactory (2). The reasons of this poor prognosis are diverse (2, 3) including lack of specific symptoms leading to late presentation, difficulties in early diagnosis, recurrence and metastasis, and the emergence of chemotherapy resistance. Hence, there is a pressing clinical and research need to explore and recognize molecular mechanisms and critical molecules involved in gastric cancer progression and metastasis and chemotherapy drug resistance.

Activated leukocyte cell adhesion molecule (ALCAM) or otherwise known as CD166, is a cell adhesion molecule that belongs to the immunoglobulin superfamily (4, 5). It is expressed in multiple cell types in the body and, as a transmembrane protein, confers homotypical and heterotypical adhesions between the cells (6). Whereas ALCAM-ALCAM interaction occurs in the same cell and between different cells, it offers adhesion between tumour-tumour cells, tumour-endothelial cells, and tumour interaction with other cells (6, 7). ALCAM can also interact with other molecules on different cells. The heterotypical interaction partners of ALCAM include

Table I. *Primers used in the study.*

Target	Forward primer	Reverse primer
ALCAM (PCR)	TTATCATACCTTGCCGATT	GGGTGGAAGTCATGGTATAG
GAPDH (PCR)	GGCTGCTTTTAACTCTGGTA	GACTGTGGTCATGAGTCCTT
ALCAM (qPCR)	CAGGAGGTTGAAGGACTAAA	ACTGAACCTGACCGTACAGGGATCAGTTTTCTTTGTCA*
GAPDH (qPCR)	AAGGTCATCCATGACAACTT	ACTGAACCTGACCGTACAGCCATCCACAGTCTTCTG*

*The underlined sequence represents z sequence.

CD6, CD9, CD44 and certain member of the integrin family proteins (5, 8, 9). This interaction is commonly seen in the interactions between tumour cells and lymphocytes and immune cells, which strongly and to some degree exclusively express CD6. The role of ALCAM in cancer has been well explored (10). At the cellular level, ALCAM can mediate tumour-endothelial interactions, thus facilitating tumour vascular embolism (11). In the tumour microenvironment, ALCAM may mediate tumour cell seeding and facilitate metastasis (12, 13). Beyond its role as a cellular protein, ALCAM can be shed from the cell surface by ectodomain cleavage by proteinases such as ADAM17 and MMP14, thus giving rise to a circulating soluble form of ALCAM (sALCAM) (14). This soluble form may act as an antagonist of the membrane bound mature ALCAM (11). Beside the biological impact of ALCAM on cancer cells, ALCAM may also have an impact on the response of cancer cells to chemotherapies in myeloma (15), pancreatic cancer (16) and recently gastric cancer (17).

Clinically, the role of ALCAM in solid tumours has been well investigated over the past two decades. It has been shown that ALCAM has two facets in different cancer types. Overall, ALCAM tends to act as a tumour suppressor in endocrine related cancers including breast cancer, prostate cancer, thyroid cancer, and potentially pituitary tumours. However, ALCAM seems to promote other solid tumour types, notably pancreatic cancer, colorectal cancer and squamous cell carcinoma (10). Studies on the role of ALCAM in gastric cancer are limited. A study with limited number of patients has shown that ALCAM protein is increased in gastric tumours compared with normal tissues and patients with gastric cancer have increased levels of soluble ALCAM (18). Work by Ishigami *et al*. (19) showed that the expression of ALCAM in gastric cancer patients was associated with significantly shorter survival and related to nodal involvement and vascular invasion.

The present study aimed to investigate the expression pattern of ALCAM, at transcript and protein levels, in clinical gastric cancer and its relationship with patient clinical outcomes. We also explored whether the expression of ALCAM was associated with response of patients with gastric cancer to neoadjuvant chemotherapies. In the light of the findings that ALCAM expression was associated with patient's response drug treatment, cell models were generated with differential expression of ALCAM and tested their responses to chemotherapeutic drugs.

Materials and Methods

Cell lines and key materials. The human gastric cancer cell lines AGS and HGC27 were purchased from ECACC (European Collection of Animal Cell Culture, Salisbury, UK). The cells were routinely maintained in DMEM-F12 supplemented with 10% foetal calf serum and antibiotics (penicillin at 100 unit/ml and streptomycin at 100 μg/ml), in an incubator at 37˚C with 5% CO_2.

An experimental plasmid containing ALCAM-targeted shRNA and a control plasmid containing scramble sequence were purchased from VectorBuilder (Chicago, IL, USA) as previously reported (11, 13). Gastric cancer cells were transfected with the plasmids to establish ALCAM knockdown cells lines. Fugene HD (Promega, Southampton, UK) transfection reagent, which was a novel, non-liposomal transfection reagent designed to transfect DNA into a wide variety of cell lines with high efficiency and low toxicity, was used in accordance with manufacturer's instructions. Following transfection, cells were subject to selection with 2 μg/ml puromycin (Fisher Scientific UK, Leicestershire, UK), prepared in growth medium. Once sufficient cell death had occurred, cells were taken out of selection and grown in maintenance medium containing 0.2 μg/ml puromycin.

Clinical cohorts of gastric cancer tissues. The present study employed two independent clinical gastric cohorts as we previously reported (20, 21). One cohort contained 316 gastric cancer tissue specimens of which 175 cases also had matched normal tissue specimens. It was used to assess the expression profile of ALCAM and its association with clinical, pathological, and clinical outcomes of the patients. The other cohort (n=86) additionally had information on patients' response to neoadjuvant chemotherapies. Both cohorts were collected with the same protocol and tissues were collected immediately after surgical resection of the gastric tumours. The cohorts were collected under a local research ethics committee approval (ethics number 2006021) with patients' consent.

The clinical cohort analysis was supplemented with information available through TCGA online datasets. The TNM plot and KM plot websites and resources were accessed to further explore the clinical significance of ALCAM in gastric cancer and its implication in patients' survival.

RNA extraction from cells and tissues, PCR, and quantitative PCR. RNA extraction was carried out using the TRI Reagent (Sigma-Aldrich, Dorset, UK). For tissues, this was carried using a

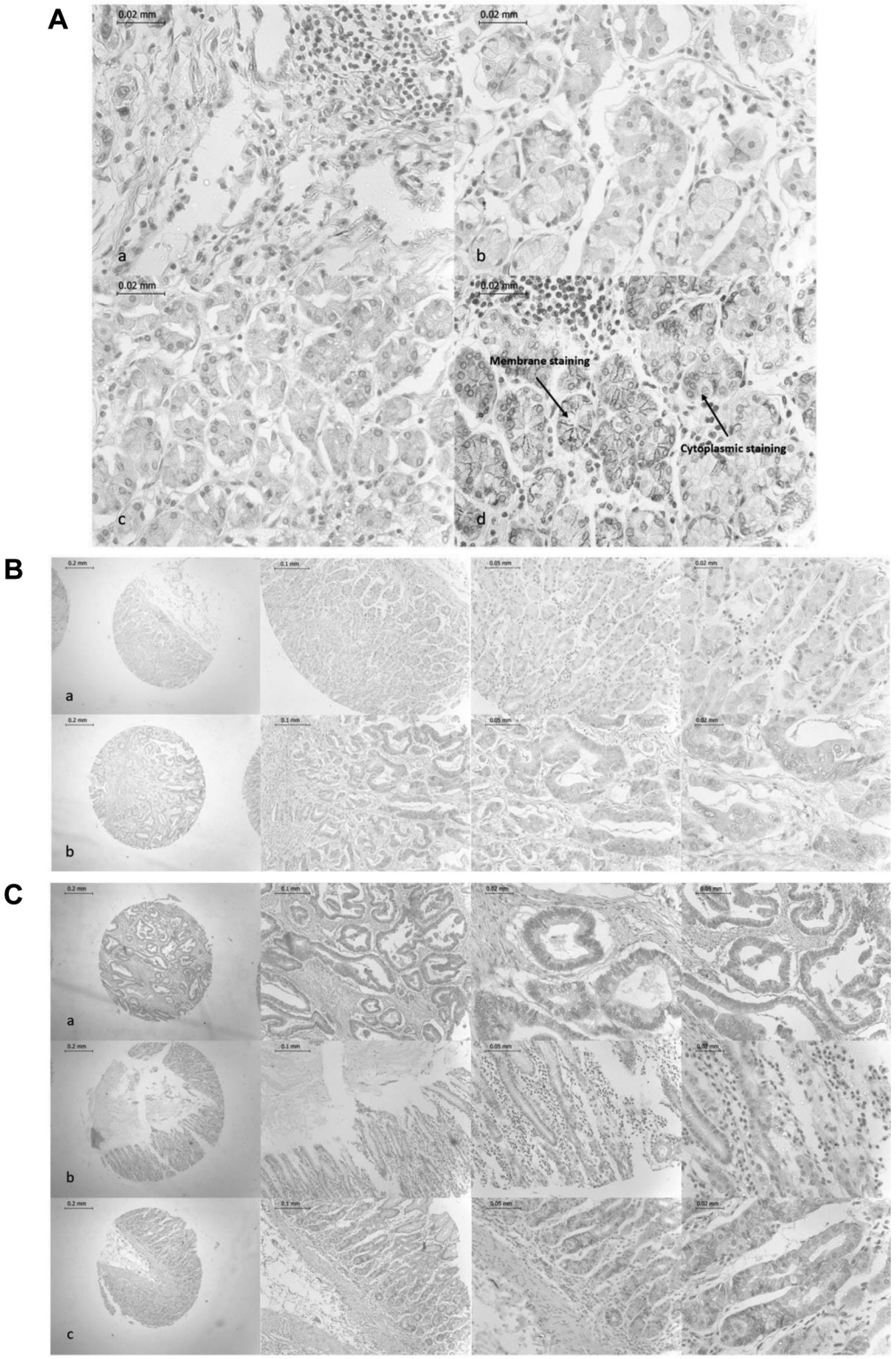

Figure 1. *Immunohistochemical staining of ALCAM in gastric cancer and normal stomach tissues. A) Representative images of varying intensities of ALCAM staining in gastric cancer TMA. a: negative staining (0); b: weak staining (1); c: moderate staining (2); d: strong staining (3). B) Representative images of ALCAM staining in adjacent normal tissues and gastric cancer tissues. a: normal tissues; b: gastric cancer tissues. C) Representative images of ALCAM staining in gastric cancer tissues with different pathological grades. a: G1-G2; b: G2; c: G3.*

Table II. *The score of ALCAM staining in gastric cancer TMA.*

		n	Intensity				Chi value	*p*-Value
			0	1	2	3		
Type	Tumour tissues	30	21	5	3	1	8.03	0.045
	Normal adjacent tissues	30	11	7	11	1		
Grade	G1-G2	2	1	1	0	0		
	G2	12	8	1	2	1	1.821	0.61
	G3	16	10	4	1	1		

homogeniser (Cole Parmer, Cambridgeshire, UK). The same concentrations of RNA from cells and tissues were used to produce cDNA using a GoScript™ reverse transcription mix, Oligo (dT) kit (Promega, Southampton, UK) in accordance with the manufacturers' guidelines. The levels of the ALCAM in cells and tissues were determined using qPCR. The chemistry used here was a molecular beacon based Amplifluor™ Uniprimer Universal qPCR system (Intergen Inc., Oxford, UK). The system is characterised by the integration of a Z sequence (5'-ACTGAACCTGACCGTACA-3') to the FAM-tagged Uniprimer probe (Table I). The reaction and detection were carried out using a StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, Leicestershire, UK). 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 of the cells and tissues.

Immunohistochemical detection of ALCAM protein on a tissue microarray (TMA). This was carried using a human tissue microarray purchased from US Biomax, Inc., (Derwood, MD, USA). The gastric cancer TMA (HStm-Ade090PG-01) was first processed for antigen retrieval in 0.1 M EDTA buffer, heated in a microwave for 20 min. This was followed by blocking the non-specific binding in 5–10% horse serum for two hours. The primary anti-ALCAM primary antibody (2 μg/ml; Novacastra, Milton Keynes, UK) was applied to the array overnight. Following extensive washing, the TMA was incubated with secondary and tertiary reagents from a Vectastain Elite Universal ABC kit (Vector Laboratories Ltd., Peterborough, UK), in accordance with the manufacturer's guidelines. The TMA was then developed with diaminobenzidine (5 mg/ml; Sigma-Aldrich, Dorset, UK) for 10 min, counterstained with Gill's haematoxylin (Vector Laboratories Ltd., Peterborough, UK), dehydrated, cleared in xylene, and mounted in DPX (Sigma-Aldrich, Dorset, UK). Once dried, sections were viewed under the microscope and digital images were captured and scored. The evaluation of the staining was using a method we recently reported (11).

Gastric cancer cell's response to chemotherapeutic drugs. Cells were seeded into 96 well plates, treated with serially-diluted drugs, and then incubated in suitable conditions. The concentrations of the drugs were chosen based on their known IC_{50} and previous studies. After 72 h, the cells were fixed with 4% formalin, stained with 0.5% crystal violet and extracted with 10% acetic acid after washing. The absorbance was measured at 595 nm using a spectrophotometer to detect cell densities. The percentage drug toxicity was calculated using the following formula: Percentage drug toxicity=(Absorbance in untreated well - Absorbance in drug treated well)/Absorbance in untreated well. The scatter plots of percentage toxicity and drug concentration were plotted, and fitting curves were used to calculate the IC_{50} value for the drugs.

Statistical analysis. Mann–Whitney *U* tests or Kruskal–Wallis tests were used to compare expression between patient groups and were undertaken using Minitab (version 14; Minitab Ltd, Coventry, UK). Two sample *t*-test and Kaplan–Meier survival analysis were performed using the SPSS statistical software (version 27; SPSS, Chicago, IL, USA). The drug response in relationship to ALCAM expression was analysed using the ROC method on SPSS.

Results

ALCAM protein staining in gastric cancer tissue. A gastric cancer TMA (HStm-Ade090PG-01) was used to examine the expression patten of ALCAM in both gastric cancer and normal tissues. In normal tissues, the staining varied between different patients. Where the staining can be assessed, the areas of staining were seen both as membrane and cytoplasmic staining (Figure 1A). It is interesting to note that normal gastric epithelial cells displayed more prominent intercellular staining (Figure 1B). In gastric cancer tissues, however, the staining was more cytoplasmic whereas intercellular staining appeared to be weakened. The intensity of ALCAM was then scored as follow: 0. Negative staining; 1. Weak staining; 2. Moderate staining; 3. Strong staining, based on an established method reported previously (22). As shown in Table II, gastric cancer tissues had significantly lower levels of ALCAM staining compared to normal adjacent tissues (p=0.045). However, no statistical significance could be found between tumour tissues with different pathological grades (Figure 1C).

ALCAM transcript expression in gastric cancer tissues. ALCAM transcript expression was analysed in both gastric cancer cohorts. A total of 316 gastric cancer patients were included in the first cohort. As shown in Table III, there was a significant difference in ALCAM transcript levels between normal and tumour gastric tissues (p=0.003). The was no

Table III. *ALCAM transcript expression in adjacent normal and gastric cancer tissues in the first cohort.*

	Variable	n	Median	Q1	Q3	*p*-Value
Tissue type	Tumour	316	6.5	0	476	0.003
	Normal	182	67.6	0	635	
Sex	Male	226	10.2	0	461	0.971
	Female	90	2.8	0	553	
T stage	T1	16	51.8	0	3,568	0.498
	T2	25	0.4	0	5,460	
	T3	41	2.7	0	187	
	T4	226	8.6	0	510	
	T1+T2	41	4.0	0	3,261	0.733
	T3+T4	267	7.0	0	408	
Lymph nodes	N0	70	5.0	0	2,045	0.526
	N1	47	0.3	0	179	
	N2	61	10.8	0	205	
	N3	132	10.2	0	599	
	N1+2+3	240	6.4	0	307	
Metastasis at diagnosis	M0	275	5.3	0	483	0.527
	M1	40	13.3	0	468	
TNM staging	TNM1	25	40.4	0	7,264	0.910
	TNM2	59	2.7	0	1,925	
	TNM3	214	8.0	0	260	
	TNM4	9	0.4	0	889	
Differentiation	High	1				
	High/moderate	6	73.6	0	2,758	0.970
	Moderate	61	0.8	0	1,948	
	Moderate/low	81	5.7	0	524	
	Low	134	10.2	0	370	
Invasion	Whole layer	227	10.8	0	546	0.221
	Subserosa	36	1.3	0	40	
	Muscular layer	30	10.3	0	12,848	
	Mucosa	11	0.2	0	81	
Embolism	No	150	12.8	0	965	0.306
	Yes	152	4.8	0	285	
Radical surgery	No	69	16.1	0	1,287	0.060
	Yes	243	2.7	0	265	
Clinical outcomes	Alive	133	1.7	0	586	0.476
	Died	180	10.8	0	443	

significant difference regarding sex, T stage, lymph nodes involvement, metastasis, TNM staging, differentiation degree, tumour invasion, tumour vascular embolism, radical surgery, and clinical outcomes, between normal and tumour gastric tissues.

Similar to the first cohort, significantly higher levels of ALCAM were observed in normal tissues compared with tumour tissues in the second cohort ($p<0.001$, Table IV). Tumours which were more than 50 mm in diameter had higher ALCAM expression compared with those less than 50 mm ($p=0.004$). No significant difference was found regarding sex, T stage, tumour location, Bormann staging, surgical approaches (D1/D2), differentiation, tumour vascular invasion, tumour size, T stage, lymph nodes involvement, TNM staging, gastric cancer related incidence, and clinical outcomes between normal and tumour gastric tissues.

ALCAM transcript expression and the clinical outcome of the patients. The survival of gastric cancer patients was also analysed. The cohorts were divided into ALCAM high expression and low expression groups based on their respective ROC best cut-off value. As shown in Figure 2, patients with low levels of ALCAM had significantly longer overall survival (OS) compared with those with high ALCAM expression in the first cohort (55.9±3.5 *versus* 43.4±2.9 months, $p=0.043$). The results were similar in terms of disease-free survival (DFS), namely high ALCAM expression patients tend to have shorter DFS (55.1±3.6 *versus* 41.9±2.7 months, $p=0.038$).

In the second cohort in which patients received neoadjuvant chemotherapy treatment (Figure 3), the OS of the patients who had low ALCAM expression was also longer compared with the high ALCAM expression group (47.5±4.9 *versus* 22.8±3.1 months, $p=0.006$) (Figure 3, left).

Table IV. *ALCAM transcript expression in adjacent normal and gastric cancer tissues in the second cohort.*

	Variable	n	Median	Q1	Q3	*p*-Value
Tissue type	Tumour	87	1.5	0	312	<0.001
	Normal	87	1,312.3	198	11,165	
Sex	Male	63	1.3	0	98	0.260
	Female	24	6.7	0	529	
Location	Proximal	31	2.5	0	45	0.550
	Distal	46	1	0	525	
	Whole stomach	7	17.9	1	14,966	
Bormann staging	1	3	1	0	45.1	0.530
	2	13	5.5	0	178	
	3	35	2.1	0	1,292	
	4	9	0.5	0	4	
Differentiation	Differentiated	20	9.3	0	503	0.436
	Undifferentiated	56	1	0	101	
	Others	8	1.4	0	358	
Vascular invasion	No	41	1.9	0	389	0.268
	Yes	43	0.8	0	193	
D2 Surgery	No	21	2.7	0	422	0.550
	Yes	66	1.1	0	284	
Tumour size	T>50 mm	42	4.5	0	986	0.004
	T<50 mm	35	0.4	0	17	
T-stage	T1-2	8	0.7	0	10.3	0.198
	T3-4	78	2.1	0	525	
Node involvement	No	16	0.7	0	465	0.474
	Yes	71	2	0	312	
TNM staging	TNM1	4	1.26	0.49	10.27	0.400
	TNM2	14	3.2	0	826	
	TNM3	45	2.2	0	505	
	TNM4	23	1.1	0	260	
GC related incidence	Free	59	2.1	0	533	0.157
	With incidence	27	0.4	0	98	
Clinical outcomes	Alive	31	1.5	0	18	0.553
	Died of GC cancer	56	1.9	0	584	

Table V. *IC_{50} of chemotherapy drugs in control and ALCAM knockdown groups of different gastric cancer cell lines.*

Cell lines	Drugs	Mean±SD		
		Control group	ALCAM-KD group	*p*-Value
HGC 27	Cisplatin	12.090±3.645	4.607±1.447	0.048
	Oxaliplatin	0.433±0.091	0.360±0.147	0.504
	5-Fluorouracil	11.107±0.702	8.017±1.706	0.044
	Paclitaxel	0.100±0.019	0.104±0.009	0.721
AGS	Cisplatin	10.967±3.461	1.207±0.561	0.009
	Oxaliplatin	8.527±0.477	6.553±0.152	0.306
	5-Fluorouracil	1.753±0.952	0.857±0.380	0.204
	Paclitaxel	0.009±0.003	0.025±0.016	0.175

The same results were observed regarding DFS (40.5 *versus* 20.3 months, p=0.020) (Figure 3, right).

ALCAM expression and patients' responses to drug treatment. We further explored whether ALCAM expression in gastric cancer was associated with patient's responses to drug treatment, namely neoadjuvant chemotherapies. This study was performed in the second cohort in which patients were evaluated for their response to treatment. As shown in Figure 4A, tumour tissues from

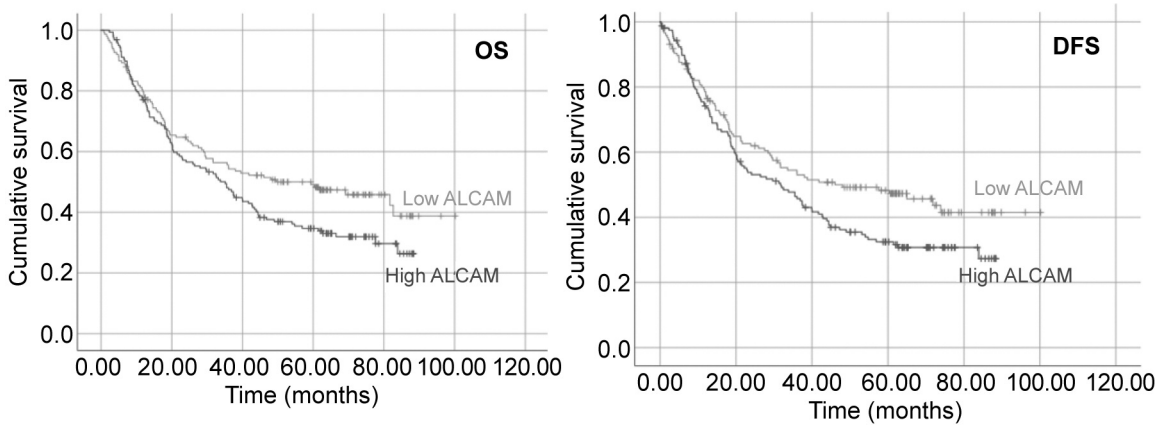

Figure 2. *The overall survival (OS) (left) and disease-free survival (DFS) (right) curves of high/low ALCAM expression groups in the first cohort of gastric cancer patients.*

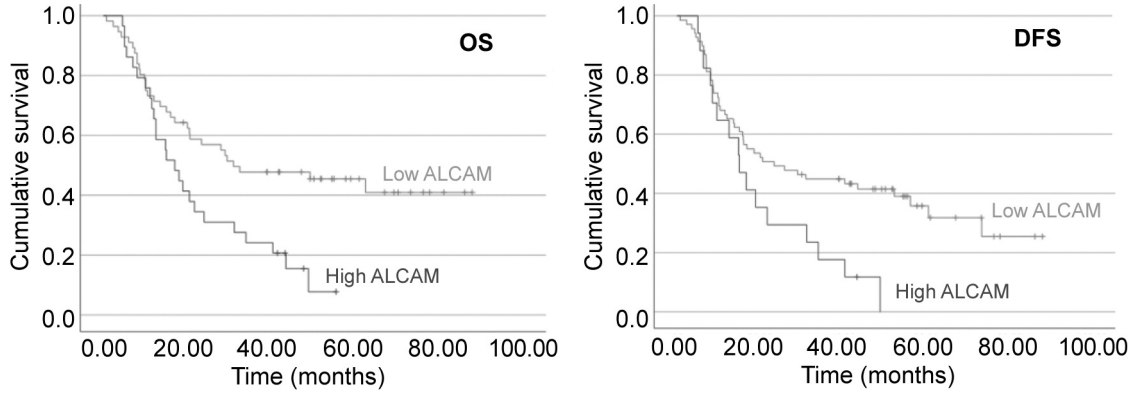

Figure 3. *The overall survival (OS) (left) and disease-free survival (DFS) (right) curves of high/low ALCAM expression groups in the second cohort of gastric cancer patients.*

patients who resisted the neoadjuvant therapies had marginally significantly higher levels of ALCAM than those responded (p=0.056, Figure 4A). Patients who resisted neoadjuvant chemotherapies had a significantly poorer OS than those who responded (p=0.013, Figure 4B) and indeed a poor DFS (p=0.017, Figure 4C). It was very interesting to note that this relationship was particularly prominent in patients with tumours of low levels of ALCAM (Figure 4D), in that patient's OS was far more sensitive to their response to neoadjuvant chemotherapies (64.5±6.8 months *versus* 32.1±8.1 months, p=0.004). However, the survival of patients with gastric tumours expressing high levels of ALCAM, was not different between those responded and resisted to neoadjuvant chemotherapies (41.2±8.9 months *versus* 36.5±5.6 months, p=0.581, Figure 4E). The same results were also observed with DFS (Figure 4F and G).

ALCAM expression of gastric cancer in TCGA database. The above survival analysis showed that patients with lower levels of ALCAM had longer OS and higher chance to response to neoadjuvant chemotherapy, which indicated that ALCAM may acted as a poor prognostic factor in gastric cancer. However, the ALCAM expression levels in gastric cancer patients seemed to be lower than that in normal patients according to the results of IHC staining and our clinical cohort data. Hence, we used TCGA database to further explore the effect of ALCAM in gastric cancer and its clinical outcomes. As shown in Figure 5, there was a significant decrease of ALCAM expression in gastric cancer tissues compared with normal tissues (p<0.001). In contrast, patients with lower levels of ALCAM seemed to have shorter OS and post-progression survival (PPS), although no statistical significance was reached (Figure 6, p=0.29 and 0.23, respectively). The TCGA data was largely in line with

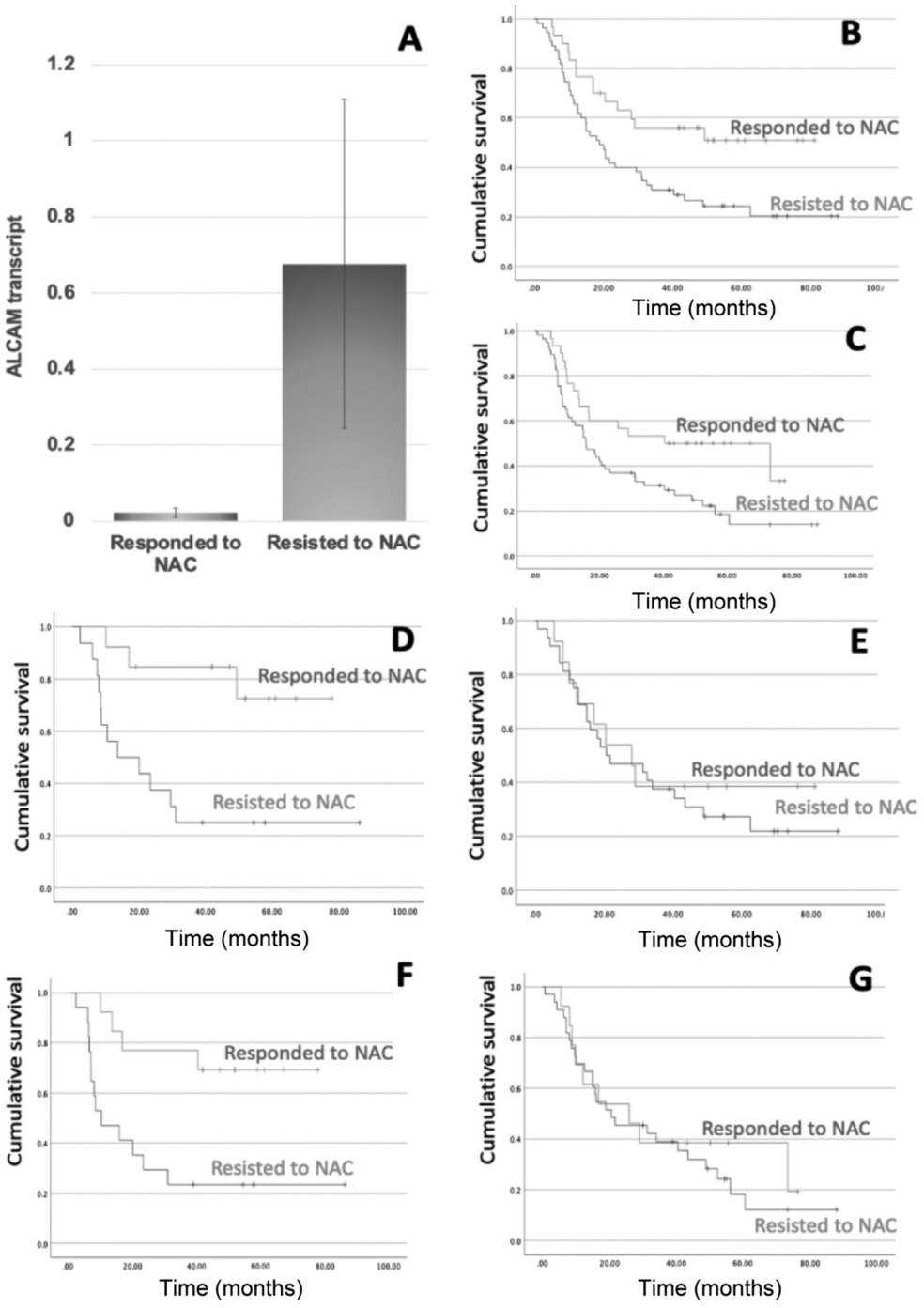

Figure 4. *ALCAM expression, patient's response to neoadjuvant chemotherapies and the clinical outcomes. A) Levels of ALCAM expression in gastric tumours from patients who responded and resisted neoadjuvant chemotherapies; patients who resisted the treatment had marginally significant higher levels of ALCAM than those who responded (p=0.056). B and C) Patient response to neoadjuvant chemotherapies and the clinical outcome as shown by OS (B) and DFS (C). Patients who resisted to therapies had a significantly shorter OS (p=0.013) and DFS (p=0.017), compared with those who responded. D and E: OS of the patients who responded and resisted to neoadjuvant chemotherapies, stratified by expression of ALCAM. Patients who had low ALCAM expression and resisted to therapies had poorer outcome than those who responded (p=0.004) (D), whereas patients who had high ALCAM expression showed no significant difference in their OS irrespective of their responses to neoadjuvant chemotherapies (p=0.0581). F and G: DFS of the patients according to their response to neoadjuvant chemotherapies and stratified by expression of ALCAM. Patients who had low ALCAM expression and resisted to therapies had the poorer DFS than those who responded (p=0.006) (F), whereas patients who had high ALCAM expression showed no significant difference in their survival irrespective of their responses to neoadjuvant chemotherapies (p=0.527) (G). Survival curves were drawn using the Kaplan–Meier method and p-value was calculated using the log-rank test.*

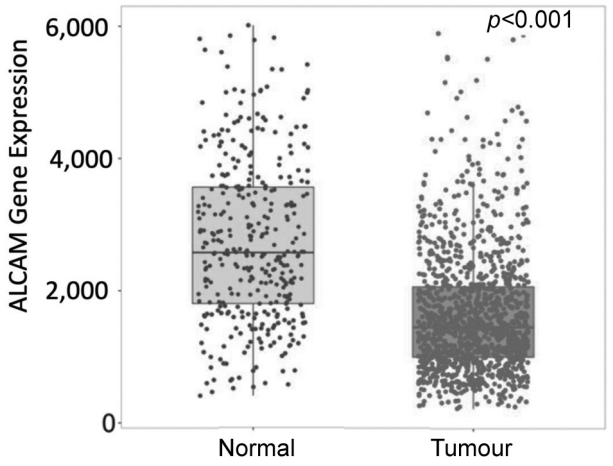

Figure 5. *ALCAM gene expression in normal (left) and gastric cancer tissues (right) from TCGA database. Gastric cancer group had significantly lower ALCAM expression compared with the normal group (p<0.001). Data analysis was conducted, and images obtained from the TNM plot website.*

our above results, namely higher levels of ALCAM expression led to poor clinical outcomes in gastric cancer patients, while opposite results were obtained when it was used as a diagnosis factor. This suggested a complex role of ALCAM in the regulation of cancer progression.

Generation of ALCAM gene-manipulated cell models. Since the association between ALCAM expression, patients' responses to treatment and clinical outcome have been indicated in the previous sections, we further explored if such association could be demonstrated *in vitro*. Here, we created ALCAM knockdown cell models using the gastric cancer cell lines AGS and HGC27. The transfection efficiency was verified by PCR. As shown in Figure 7, both AGS and HGC27 showed a clear reduction of ALCAM expression following ALCAM knockdown, demonstrating the reliability of the gastric cancer cell models.

Drug toxicity assays based on ALCAM gene-manipulated cell models. To validate our clinical findings on the relationship between ALCAM expression and patients' responses to drug treatment, we performed drug toxicity assays using ALCAM knockdown cell models as mentioned above. Figure 8 and Figure 9 show the cytotoxicity curve of AGS and HGC27 cell lines treated with four representative chemotherapy drugs: Cisplatin, Oxaliplatin, 5-Fluorouracil and Paclitaxel. The ALCAM knockdown cells appeared to be more sensitive to Cisplatin, Oxaliplatin and 5-Fluorouracil compared with their respective control cells, whereas in the Paclitaxel-treated group, the control and ALCAM knockdown cells did not exhibit significant difference.

As shown in Table V, the IC_{50} of Cisplatin in HGC27 ALCAM knockdown cells was significantly lower than that in HGC27 control cells (12.090±3.645 μM *versus* 4.607±1.447 μM, p=0.048). In the HGC27 cell line, 5-Fluorouracil was found to have a reduced IC_{50} following ALCAM knockdown (11.107±0.702 μM *versus* 8.017±1.706 μM, p=0.044). In the AGS cell line, Cisplatin had lower IC_{50} in ALCAM knockdown groups compared with AGS control cells (10.967±3.461 μM *versus* 1.207±0.561 μM, p=0.009). Although the rest of the groups showed the same trend according to our hypothesis, they did not reach statistical significance.

Discussion

The present study presented the findings on the relationship between ALCAM expression and the clinical, pathological, and clinical outcome of patients with gastric cancer. By employing two separate cohorts, we collectively found that ALCAM transcript is a valuable prognostic indicator for OS and DFS. The study also demonstrated that the levels of ALCAM transcript is a useful indicator for evaluating patient's response to neoadjuvant chemotherapies, and this is supported by *in vitro* cell model-based investigations. It was very surprisingly to discover that ALCAM and patient's response to neoadjuvant therapies together identified patients with the poorest clinical outcome.

Neoadjuvant chemotherapy before surgery is administered for "downstaging and downsizing" locally advanced gastric tumours. It has been shown to increase the chance for curative resection, reduce micro metastases in early stages, and allow an *in vivo* response assessment of treatment (23, 24). However, not every patient would benefit from preoperative drug treatment due to the responsiveness of chemotherapy drugs, side effects of the treatment and the postponement of surgery timing. Hence, surgeons should fully evaluate patients' conditions beforehand and perform neoadjuvant chemotherapy with caution. It is clear from the present study that when patients had high levels of ALCAM in gastric tumours, neoadjuvant chemotherapies did not result in additional benefit whether patients responded or resisted to the treatment. However, when gastric tumours had low levels of ALCAM, responders to neoadjuvant chemotherapies experienced huge survival benefits, measured by both OS and DFS, compared with those who resisted. Thus, this interesting finding has clinical significance but needs to be interpreted with reasonable caution due to the size of the present study. It is thus strongly argued for a larger and ideally prospective study to further validate this finding.

Previous studies have focused on the correlation between ALCAM, the survival of cancer patients and chemotherapy resistance. Hong and colleagues (16) reported that silencing of ALCAM via siRNA could reduce cell adhesion and induce chemoresistance in pancreatic cancer cells. A study by Zhou

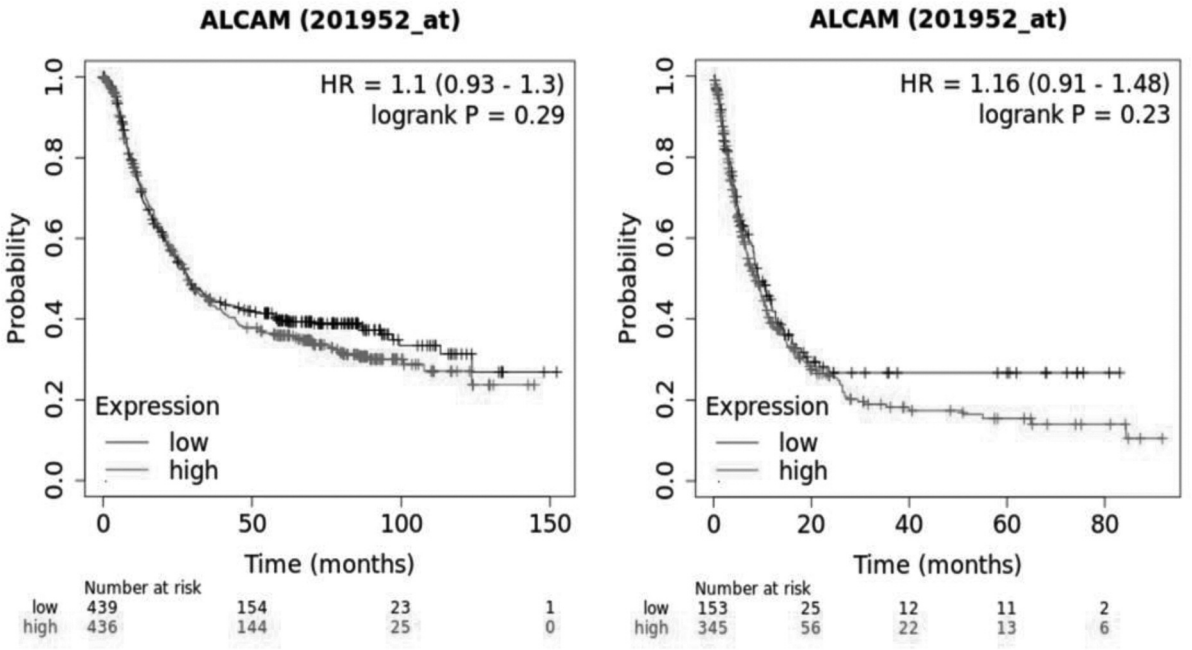

Figure 6. *The overall survival (OS, left) and post-progression survival (PPS, right) of low and high ALCAM expression groups in gastric cancer patients from TCGA database. Data analysis was conducted, and images obtained from the KM plot website. ROC cut-off value was used as the cut-off point of the cohorts. Survival analysis was performed by Kaplan–Meier model.*

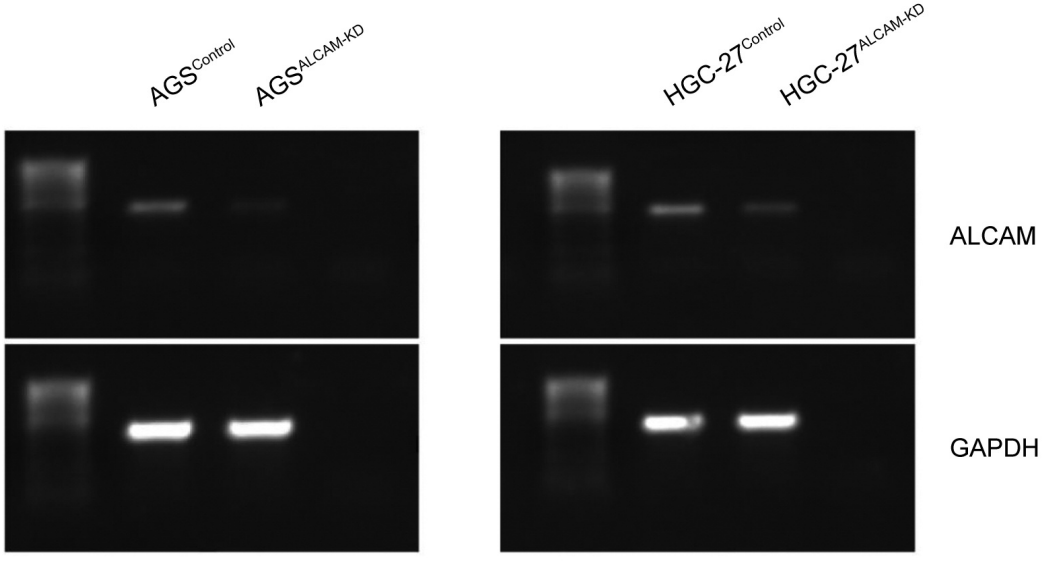

Figure 7. *The ALCAM transcript expression in ALCAM knockdown cell models as detected by PCR.*

et al. (25) showed that ALCAM-positive giant cell tumours of bone (GCTB) exhibited increased resistance to chemotherapy-induced cell death, and ALCAM expression was associated with clinical outcomes of patients with GCTB. In non-small cell lung cancer (NSCLC), EpCAM+/ALCAM+/CD44+ cells were found to have higher alkaline dehydrogenase (ALDH) activity and relatively higher resistance to both 5-fluorouracil and cisplatin compared to EpCAM-/ALCAM-/CD44- cells

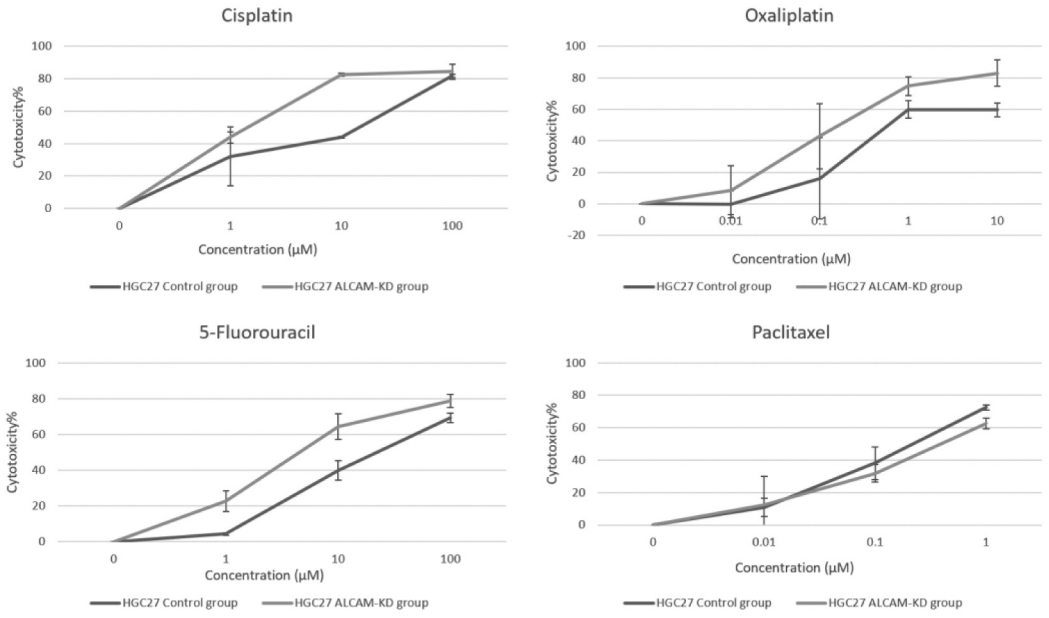

Figure 8. *Effect of ALCAM knockdown on chemotherapy drug toxicity in HGC27 cells. X-axis: drug concentration. Y-axis: percentage cytotoxicity.*

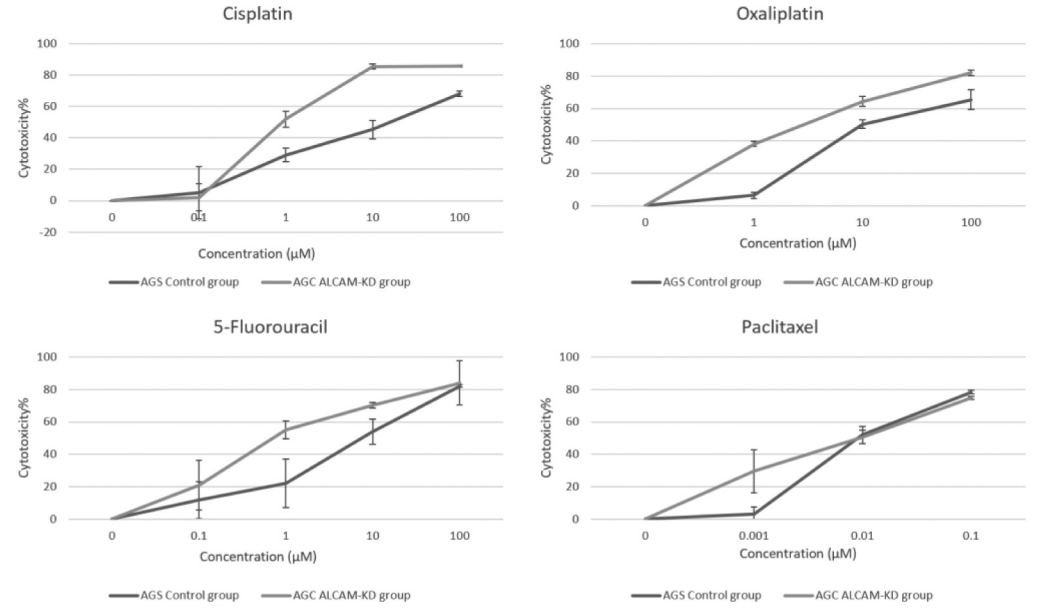

Figure 9. *Effect of ALCAM knockdown on chemotherapy drug toxicity in AGS cells. X-axis: drug concentration. Y-axis: percentage cytotoxicity.*

(26). In terms of gastric cancer, Ni *et al*. (17) conducted magnetic-activated cell sorting and successfully isolate CD133+/CD166+ cell populations from the gastric cell lines BGC-823 and SGC-7901. The CD133+/CD166+ cells showed more malignant features, including cell proliferation, invasion, and migration. Notably, the CD133+/CD166+ gastric cancer cells were highly resistant to both cisplatin and oxaliplatin compared with other groups of cells in chemotherapy

resistance assay. These previous studies lend additional support to the present study, which showed that the levels of ALCAM in gastric cancer cells and the response of cells to chemotherapeutic drugs are also drug dependent and cell dependent. For example, two gastric cancer cell lines showed increased sensitivity to cisplatin and 5- fluorouracil following ALCAM knockdown. This was not observed with paclitaxel and oxaliplatin. Together with the clinical findings, these results suggest that ALCAM expression may indeed aid the decision making in selecting suitable patients and suitable drugs for neoadjuvant drug treatment.

The present study presented limited data on the protein expression in normal gastric tissue and gastric cancer tissues. It was noted that membranous staining of ALCAM in normal epithelial cells is more prominent than that in gastric cancer tissues. Owing to the small number of available tissues for immunohistochemistry, we were unable to assess whether this pattern of protein distribution had a clinical significance. However, Ishigami *et al*. (19) assessed the immunohistochemical staining of ALCAM in 142 gastric cancer tissues, and found out that the membranous staining pattern of gastric cancer cells appears to be a useful indicator for patients with poor clinical outcomes when compared with ALCAM negative tumours. This together with the present study suggests that a more comprehensive and large analysis of protein distribution is necessary. In a separate study, Erturk *et al*. (27) showed that patients with gastric cancer had significantly higher levels of serum ALCAM, arguably the shed soluble ALCAM, than controls. Together, these results suggests that there should be more investigation into ALCAM in gastric cancer both at tissue level and in the circulation. However, the current study supports the finding that determination of ALCAM transcript represents a useful approach in assessing patient's prognosis and patients response to drug treatment in gastric cancer.

Conclusion

The present study reports that ALCAM transcript expression in human gastric cancer is a strong prognostic indicator for the clinical outcome of the patients, notably their OS and DFS. ALCAM expression is also a tentative indicator for patient's response to neoadjuvant chemotherapies and patients with low levels of ALCAM and failure to respond to neoadjuvant chemotherapies tend to have a very poor clinical outcome. Together with the *in vitro* findings, the study further demonstrates that ALCAM is an indicator for gastric cancer cell sensitivity to chemotherapy drugs.

Conflicts of Interest

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

Authors' Contributions

Conceptualization, WGJ, AJS and JJ; methodology, AJS, YY, WGJ; formal analysis, YY, FR, WGJ, AJS; investigation, YY, FR, JK, SJ, WGJ, AJS; resources, JJ, SJ, KJ; data curation, KJ, SJ, YJ, JJ, WGJ, AJS; writing – original draft preparation, YY, AJS, WGJ, FR; writing – review and editing, YY, AJS, FR, WGJ, JJ and JK; visualization, YY, FR; funding acquisition, WGJ and JJ. All Authors have read and agreed to the published version of the manuscript.

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