Death associated protein-3 (DAP3) and DAP3 binding cell death enhancer-1 (DELE1) in human colorectal cancer, and their impacts on clinical outcome and chemoresistance

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Received August 15, 2022; Accepted October 19, 2022

DOI: 10.3892/ijo.2022.5455

Abstract. Death associated protein-3 (DAP3) was identified as a responsive protein to interferon-gamma-induced cell death which possibly exerts this regulation by interacting with DAP3 binding cell Death enhancer-1 (DELE1), a newly discovered mitochondrial stress protein in response to cell stress signals. Whilst DAP3 has been shown to be aberrantly expressed in several cancer types (i.e. breast cancer), little is known about the relationship between DAP3 and DELE1 in cancers. The present study examined the expression levels of both DAP3 and DELE1 in clinical colorectal cancers (CRCs), as well as their implication on chemoresistance and mechanism behind the action. Firstly, transcript levels of both DAP3 and DELE1 were quantitatively assessed in a clinical cohort of CRC (n=94). Tumour tissues had significantly higher levels of DAP3, but not DELE1 compared with normal tissues. Levels of DAP3 and DELE1 had a significant association with patient's clinical outcomes and local recurrence. DAP3 and DELE1 significantly correlated in normal colorectal tissues but not in tumour tissues. Secondly, the protein levels of DAP3 and DELE1 were evaluated in both normal and tumour colon tissues which showed that both proteins were highly aberrant in CRC tissues. In addition, both DAP3 and DELE1 at transcript and protein levels were identified as prognostic factors for patient's clinical outcomes. Furthermore, in *in vitro* assays, knocking down DAP3 or DELE1, and in particular both DAP3 and DELE1 together rendered the CRC cells more sensitive to chemotherapy drugs, consistent with clinical findings of the TCGA-COAD datasets. The acquisition of drug sensitivity following the genetic knockdown was independent of the mitochondrial metabolism, as neither DAP3 knockdown nor DELE1 knockdown showed a significant change. In summary, DAP3 and DELE1 are highly aberrant in CRCs, and both molecules are prognostic factors for patient's clinical outcomes and local recurrence, and are indicators for chemoresistance.

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Key words: death associated protein-3, DAP3 binding cell death enhancer-1, colorectal cancer, survival, drug resistance

Introduction

Death associated proteins, DAPs, are a small group of proteins that appear to be responsive proteins in interferon gamma-induced programme cell death (1,2). There are two members of the DAP protein family, namely DAP1 and DAP3, which have molecular weights of 15 and 46 kDa, respectively. Whilst DAP1 has been shown to be a regulator of autophagy (3) and lost in certain malignancies including breast cancer (4), neurological tumours (5) and pancreatic cancer (6), the roles of DAP3 in cancer cells and in clinical cancer setting conditions are far from clear.

DAP3 is responsive to interferon-beta (IFN-β) and -gamma (IFN-γ)-induced anoikis and apoptosis that require IPS1 (interferon-beta promoter stimulator 1) (1,7,8). DAP3 has been shown to be highly expressed in pancreatic cancer (6), glioblastoma multiforme (9), late stage thymomas (9,10), the non-epithelial derived tumour, Burkitt Lymphoma and a subtype of acute lymphoblastic leukaemia (11). By sharp contrast, the levels of DAP3 were low in gastric (12) and breast cancer (10,13) when compared with the respective normal counterpart tissues. In addition, DAP3 in cells derived from

certain solid cancers could be used as an indicator for patients' response to drug and radiation therapies (12,14). For example, knocking down DAP3 in human lung cancer A549 and H1299 cells markedly increased the rate of cell death and reduced the fraction of cell survival in response to radiation and chemo drug treatment (14). In human hepatoma cell line Hep3B, DAP3 is one of the prominent responsive genes regulated by a P53-regulating protein TP63 (15). The reasons for discrepancies of the roles played by DAP3 in cells are not clear. One possibility is the dependency on cell types and cancer types, a phenomenon known for a few cancer-related molecules. The idea that the discrepancies may be due to DAP3 gene mutation is less convincing as there has been little evidence of mutation in the key regions of the DAP3 gene (16). A recent study has shown that in pancreatic cancer, high levels of DAP3 were associated with a significantly shorter overall survival (OS) and disease-free survival (DFS) of the patients and that there was a close relationship between DAP3 expression and lymph node involvement (6). In addition, it has been reported recently that DAP3 is able to mediate the variant splicing event via forming substrate-specific splicing inducing ribonucleoprotein complexes and by modulating splicing factors to cause indirect effect on splicing (17), a possible reason for DAP3 to contribute to the poor outcome of patients.

Previously, a protein that interacts with DAP3 protein has been reported and named DELE1 (DAP3 Binding Cell Death Enhancer 1, also known as KIAA0141). DELE1 has been identified by a yeast two-hybrid screening in HeLa cells (18). DELE1 overexpression has been shown to render cell apoptosis in response to tumour necrosis factor and TRAIL (18). By interacting with DAP3, DELE1 may coordinate a cell death event in cells as silencing DELE1 would reduce death receptor (DR)-mediated apoptosis. DELE1 protein contains a mitochondrial targeting sequence at the N-terminus and two Tetratricopeptide Repeat Motifs (TPR) motifs in protein-protein interaction domains (18), and was subsequently found to be a key component, together with Overlapping Activity With M-AAA Protease (OMA1) and Heme-Regulated Eukaryotic Initiation Factor EIF-2-Alpha Kinase (HRI) in mitochondria stress signalling pathways (19-21). It has been reported that DAP3 knockdown results in mitochondria fragmentation (14,22). These findings suggested that DAP3, by interacting with DELE1, regulates cell functions and cell death via the mitochondria signalling pathways. DAP3 and DELE1 are important growth/death regulators of cancer cells and have important clinical values. However, the roles played by the two molecules markedly vary depending on cell and tumour types. Along this line, only very limited tumour types have been investigated. In addition, investigation on both DAP3 and DELE1 together in a single clinical cancer type has not been conducted.

Colon cancer is one of the leading cancer types globally and ranks the fifth in both new cases ($1.15x10^6$, 6.0% of total) and number of deaths ($0.58x10^6$, 5.8% of total) (23). Its incidence and death rate are higher in countries with high human development index compared with those with medium/low indexes. Whilst surgery, if the tumour is discovered early, remains an important option for colon cancer treatment, chemotherapies and radiation therapies are essential for late-staged colon cancer patients. However, there are limited chemotherapeutic agents for colon cancer patients, commonly used including fluorouracil, oxaliplatin, capecitabline (Xeloda) and Irinotecan, which are often used in combinations. With the recognition of the importance of angiogenesis in the development and progression of colon cancer, anti-angiogenic therapies are also available for these patients; for example, the anti-VEGF humanised antibody therapy, Avastin/Bevatuzumab, has become a choice for patients with colorectal cancers (CRCs). Despite all the latest options of treatment, survival of the patients, particularly those in late state remains very poor, namely 90, 80, 70 and 10% for stage 1, 2, 3 and 4 tumours, respectively. Amongst a wide range of studies on the factors linked to the outcome of the patients, a recent study from the US has indicated that the clinical outcome of colon cancer (stage-3) appeared to be independent of race, when patients of white and black ethnicity were compared, and unrelated to median household income (24), arguing that more wider factors, including biological factors, may contribute to the outcomes of the patients.

In light of the important roles of DAP3 and DELE1 in cancer cells and the limited information on their roles in clinical cancers, in the present study, the expression of both DAP3 and DELE1 in gene transcript and protein levels were investigated and their potential prognostic and therapeutic value in human CRC was explored. In the present study, for the first time to the best of our knowledge, it was reported that both DAP3 and DELE1 were overexpressed in colon cancer and that their high levels serve as a significant indicator for the clinical outcomes. Furthermore, silencing DAP3 and DELE1 or DAP3/DELE1 together in colon cancer cell models significantly increased the sensitivity to chemotherapeutic drugs *in vitro*.

Materials and methods

Reagents. Primary antibodies used in the current study included anti-DAP3 antibody (cat. no. sc-373911) and anti-DELE1 antibody (cat. no. sc-515080; both from Santa Cruz Biotechnologies Inc.). Anti-DAP-3 antibody was a mouse monoclonal IgG1 (Kappa light chain) against human DAP3 (Synthetic peptide corresponding to Human DAP3 aa 63-95 conjugated to keyhole limpet hemocyanin; ENDPAKHGD QHEGQHYNI SPQDLETVFPHGLPPRFVMQ VKTFS), whilst anti-DELE1 antibody was also a mouse monoclonal IgG1 (Kappa light chain) against human DELE1 (Synthetic peptide corresponding to Human DELE-1 aa 338-400 conjugated to keyhole limpet hemocyanin vs. LLKQAADSGLRE AQAFLGVLFTKEPYLDEQRAVKYLWLAANNGDSQSR YHLGICYEKGLG). The secondary antibody used was goat anti-mouse IgG-HRP (cat. no. abs20001; Absin Bioscience, Inc.). Anti-human DELE1 siRNA was purchased from Santa Cruz Biotechnologies, Inc. An anti-human DAP3 ribozyme transgene that was specifically used to target and knockdown human DAP3 was prepared as previously reported (12).

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, Wales, UK) and stored at -80˚C until further processing. Patients with other cancers, family history

Table I. Primer sequences using in the qPCR analyses.

Gene name	Primer sequence 5'→3'
DAP3	F: AAAGCACTGAGAAAGGGAGT
	R: ACTGAACCTGACCGTACAACCTCTTTAGGTCTTTCAGCA
DELE1	F: GTCATGAGCATGGCAGAG
	R: ACTGAACCTGACCGTACAACCTGGCATAGCGGTACT
GAPDH	F: AAGGTCATCCATGACAACTT
	R: ACTGAACCTGACCGTACAGCCATCCACAGTCTTCTG

Underlined sequence of the primers is the Z-sequence, used in the Ampliflor-based qPCR assays. qPCR, quantitative PCR; DAP3, death associated protein-3; DELE1, DAP3 binding cell death enhancer-1.

of cancers and patients who received chemotherapies before surgery were excluded. The median age of the patients was 73 years (range 25-88 years) and the cohort had 43 female and 51 male patients. The collection was approved by the local research ethics committee Bro Taff Research Ethics Committee (Ref. 05/DMD/3562). Written informed consent was given by all patients. The clinical, pathological and outcome information were retrospectively collected after surgery and during the follow-up. Frozen sections were produced for routine histology and immunohistochemical studies, where sections were cut at 6 μm thickness, and 20 sequential sections of 20 μm thickness were collected and homogenised for RNA extraction. Reverse transcription of the tissue RNA was conducted using a RT kit (Sigma-Aldrich; Merck KGaA), by following the manufacturer's instructions.

Colon cancer cell line. Human CRC cell line, RKO, was purchased from ECACC (European Collection of Animal Cell Culture). RKO cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (both from Sigma-Aldrich; Merck KGaA) and antibiotics (penicillin and streptomycin at 100 unit/ml and 100 μg/ml, respectively). Cells were maintained at 37˚C with 5% CO_2.

Quantitative analyses of gene transcripts. Transcript levels of DAP3 and DELE1 in colorectal tissues and colon cancer cells were determined by reverse transcription-quantitative polymerase chain reaction. RNA was extracted from described experimental materials by using TRIzol® reagents (Sigma-Aldrich; Merck KGaA), following the manufacturers' instructions. RNA samples were then quantified to 500 ng/μl and were processed using a reverse transcription kit (Promega Corporation) to synthesise cDNA, by following the manufacturer's protocol. The chemistry for qPCR was based on Ampliflor Uniprimer™ (Intergen), a molecular beacon-based technology with modifications (25,26). StepOne plus systems (Thermo Fisher Scientific, Inc.) was applied in the present study for amplification and quantification. The primer sequences including those for GAPDH, DAP3 and DELE1 are listed in Table I. To one of the target specific primers, a Z-sequence, ACTGAACCTGACCGTACA-(underlined in Table I) was added at the 5' end of the primer. The Z-sequence complements the stem region of the FAM-tagged probe, Ampliflor Uniprimer™ for amplification and detection. An internal DNA standard with known quantity was included in all the assays for calculation of expression levels as previously reported (12). GAPDH was used as a house-keeping gene for a loading control.

Immunohistochemical (IHC) analysis. The IHC staining of DAP3 and DELE1 was performed using a tissue microarray (TMA) (52 cases of colon cancer and 62 cases of adjacent tissue as control). Sections were dewaxed in xylene and rehydrated through a graded series of ethanol/distilled water, ending with a final wash in PBS. Following a 2-h 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 μg/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, Inc.). Briefly, sections were incubated for 30 min with the biotinylated secondary antibody from the kit, made following the manufacturer's protocol, 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 in tap water prior to counterstaining with Gill's haematoxylin, before bluing in tap water, dehydrating in a graded series of ethanol, clearing in xylene and mounted with DPX. The staining was examined using a light microscope by independent pathologists to determine the aberrant expression of DAP3 and DELE1 in colon cancer. The ethics approval (approval no. 2022-019) for this protocol was granted by the Yuhuangding Hospital Research Ethics Committee (Yantai, China).

Implication of DAP3 and DELE1 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 DAP3 and DELE1 with patients' responses to chemotherapies was analysed at www.rocplot.com (accessed 6-11 February 2021) (28). The responses were tested by the ROC method for the chosen gene probes (208822_s_at for DAP3 and 201977_s_at for DELE1) to allow classification of the patients based on their responses to chemotherapies. The levels of DAP3 and DELE1 in the chemo-responsive and chemo-resistant groups were compared using Mann-Whitney

U test. Correlation with angiogenic markers was analysed using Spearman's correlation analysis.

DAP3 and DELE1 knockdown cell models. RKO cells were transfected by the anti-DAP3 ribozyme created in our laboratory as previously reported (29) by using the pEF6/V5-HIS TOPO TA vector (Invitrogen/ThermoFisher Scientific, Inc.) to create the DAP3 knockdown cell model. An empty circular pEF6/V5-HIS TOPO vector, obtained from Invitrogen; Thermo Fisher Scientific, Inc. was used as a transfection control. In both cases, blasticidin (2 μg/ml) was used as a selection antibiotic to create a stable transfection cell model. In addition, a DELE1 siRNA (h) (cat. no. sc-91731; Santa Cruz Biotechnologies, Inc.) was applied to silence the protein expression of DELE1 in RKO cells. In this case, a control siRNA (cat. no. sc-37007) was used as a transfection control. The dual knockdown cell models were also established by using anti-DAP3 ribozyme and DELE1 siRNA. These transfections were conducted using Lipofectamine™ 3000 transfection reagent (Thermo Fisher Scientific, Inc.), by following the instruction provided by the manufacturer. In brief, transfections were performed once RKO cells reached 70% confluence in a six-well plate. For every two transfections, solution A was prepared by mixing 15 μl of Lipofectamine™ 3000 transfection reagent with 250 μl of Opti-MEM™. Solution B was prepared by mixing Opti-MEM™ with 5 μg of plasmid or 150 pmol of siRNA to a final volume of 250 μl, and an additional 10 μl of P3000 reagent was supplemented into solution B if plasmid was used for transfection. Solution A was mixed with solution B followed by incubating for 15 min at room temperature. A total of 250 μl of the complex was then added to each well and cells were incubated at 37˚C with 5% CO_2 for 48 h. Blasticidin was used to maintain the transfected DAP3 knocked down cells at the concentration of 1 μg/ml.

Cell growth and cytotoxicity assays. Two days after transient transfection, 4 types of RKO cells (wild type, DAP3 knockdown, DELE1 knockdown and double knockdown) were harvested and seeded in each well (12,000 cells in 100 μl medium) on a 96-well-plate treated with indicated chemotherapy drugs at different concentrations. The 96-well plates were then added with serial diluted chemotherapy drugs including 5-fluorouracil (5-FU; range 0.16-100 μM), Docetaxel (DTX; range 0.1-1,000 nM) and Methotrexate (MTX; range 32-4,000 nM). 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.

Metabolic assays. Metabolic assay kits were purchased from Promega Corporation to evaluate the metabolic profile of CRC cells following the genetic modifications, which were performed according to the manufacturer's instructions. Griess Reagent System (Promega Corporation) was carried out to measure nitrite (NO_2^-) concentration to study the impact of DAP3 and DELE1 on nitric oxide levels (NO) (27). Briefly, $3x10^4$ cells of each of the generated RKO models were seeded in a 96-well plate in triplicate and incubated overnight at 37˚C with 5% CO_2. Sodium Nitrite (100 μM) was added in the 96-well plate in triplicate and 6 series of two-fold dilution were performed to create a Nitrite Standard Reference Curve. A total of 50 μl of Sulfanilamide solution was supplemented into each test sample and incubated at room temperature, avoiding light for 10 min, followed by adding NED solution (50 μl) and further incubation in the dark at room temperature for 10 min. The absorbance was then measured using a LT4500 Plate Reader (Wolf Laboratories, Ltd.) at 540 nm and normalised based on the Nitrite Standard Curve.

NAD(P)H-GloTM Detection System was used to investigate concentrations of reduced forms of nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) (Promega Corporation) (Ward and Thompson, 2012). In brief, 30,000 cells of each RKO model were seeded in a CELLSTAR® 96 well plate (Sigma-Aldrich; Merck KGaA) in triplicate, followed by incubating overnight at 37˚C with 5% CO_2. During the period of the experiment, 50 μl of NAD(P)H-GloTM Detection Reagent was prepared by mixing Reconstituted Luciferin Detection Reagent, Reductase and Reductase Substrate provided by the manufacturer together, and was supplemented into each test sample. After shaking gently, the plate was incubated at room temperature for 60 min, followed by carrying out GloMax®-Multi Detection System (Promega Corporation) to measure the luciferase signal.

To perform lactate and glucose detection assays, four types of RKO cells including wild type, DAP3 knockdown, DELE1 knockdown and dual knockdown of DAP3 and DELE1 were seeded on a 96-well-plate at the density of $3x10^4$ cells per well (three wells for each type) and incubated at 37˚C overnight. Following transfer of 2 μl of cell culture supernatant to the spare wells of the plate, the medium was diluted with 98 μl of PBS. Then 50 μl of the prepared samples were mixed thoroughly with the lactate detection reagent (Promega Corporation) at the ratio of 1:1, and the plate was further incubated at the room temperature for 60 min. GloMax®-Multi Detection System (Promega Corporation) was applied to determine the lactate in the samples quantified by luminescence recording. Another 2 μl of supernatant from the wells was transferred to the spare wells and diluted with 98 μl of PBS in the same method listed, then the diluted medium was further diluted 2-fold with PBS. After that, 50 μl of the diluted samples were transferred to a new 96-well-plate and mixed with 50 μl of the glucose detection reagent (Promega Corporation). After incubation at room temperature for 1 h, the glucose levels in these samples were determined using GloMax®-Multi Detection System (Promega Corporation) quantified by the luminescence.

Statistical methods. All statistical analyses were conducted using SPSS (version 27.0; IBM Corp.). Survival analysis was performed by Kaplan-Meier with log ranked method and Cox Regression. Correlation was determined by Spearman's correlation methods. Pairwise sample comparisons were obtained by 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 by ANOVA test followed by Bonferroni correction. $P<0.05$ was considered to indicate a statistically significant difference.

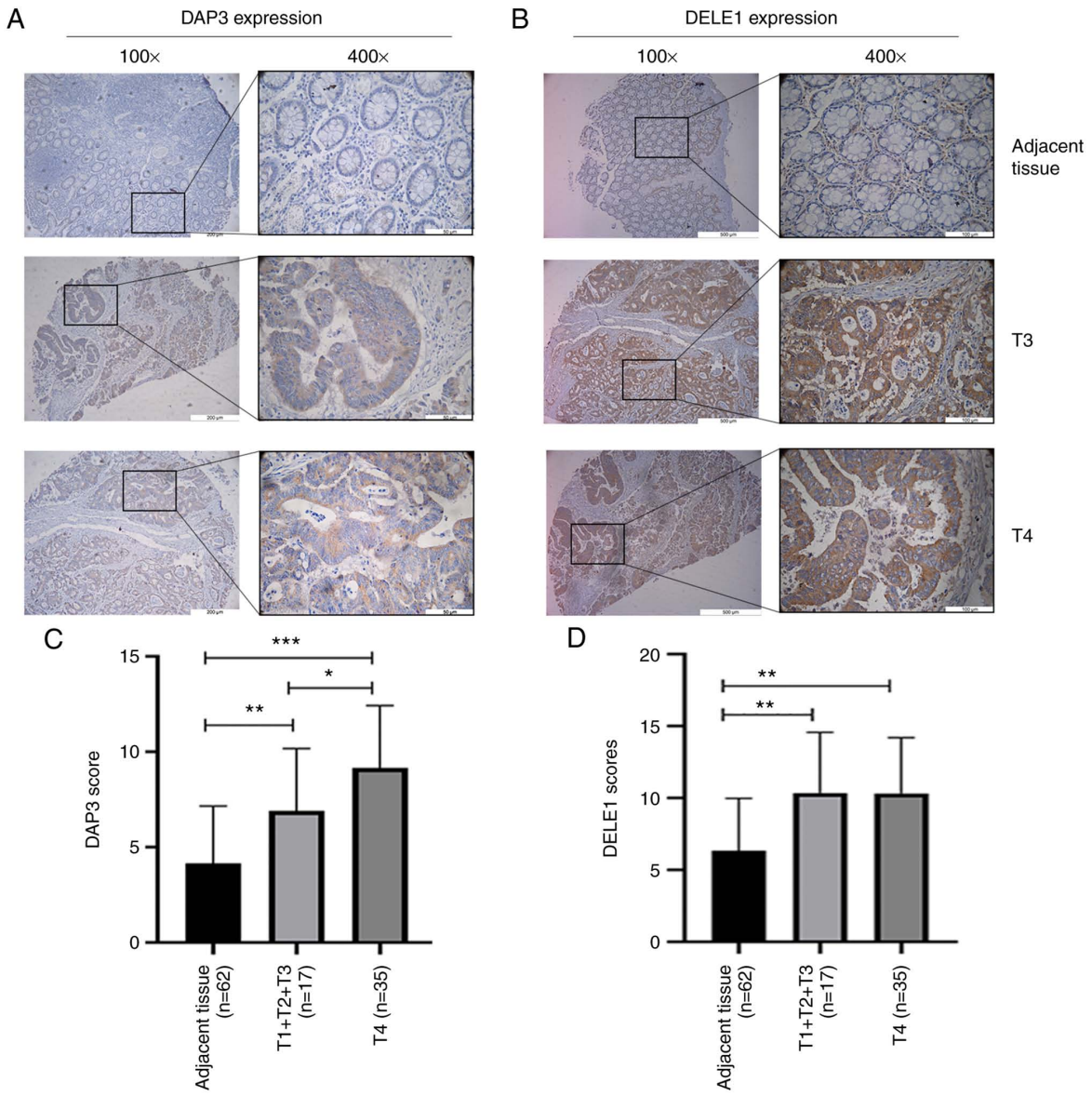


Figure 1. IHC staining for the DAP3 and DELE1 in colon cancer tissues (n=52) and the adjacent tissues (n=62). (A) DAP3 expression and (B) DELE1 expression in adjacent normal tissue, T3 and T4 colon cancer tissue with the magnification of x10 and x40, respectively. (C and D) Box plot presenting the differential (C) DAP3 and (D) DELE1 expression in adjacent tissue, early stage, and the advanced stage of colon cancer. *P<0.05, **P<0.01 and ***P<0.001. DAP3, death associated protein-3; DELE1, DAP3 binding cell death enhancer-1.

Results

Distribution of DAP3 and DELE1 protein in colorectal tissues and CRC. DAP3 staining in normal and tumour tissues. DAP3 was detected largely in the cytoplasm of cells, particularly in CRC cells. Compared with normal epithelial cells of the colon, CRC cells had marked staining in their cytoplasm. Neither normal or malignant cells showed significant staining in the nucleus (Fig. 1A and C). Additionally, neither the stromal cells nor infiltrating lymphocytes had a significant level of DAP3 staining.

DELE1 staining in normal and tumour tissues. Staining of DELE1 protein appeared to be more diverse than that of DAP3 (Fig. 1B and D). In normal colon tissues, the DELE1 staining appeared to be primarily in the cytoplasmic region of mucosal cells. Notably, basal/stem cells also showed strong DELE1-positivity in their cytoplasmic regions. There was, occasionally, the staining in the stromal region. In submucosal tissues, there were clear indications that endothelial cells (both vascular and lymphatic vessels) had DELE1-positive staining. Tumour tissues stained more prominently than normal tissues, again mainly in the cytoplasmic region of cancer cells. Moreover, there were membrane visible staining on cancer cells.

Expression and distribution pattern of DAP3 and DELE1 gene transcripts in CRC. Comprehensive analysis of DAP3 and DELE1 gene transcripts was then carried out, and it was found that tumour tissues had markedly higher levels of DAP3 and DELE1 than normal tissues (P=0.007 and P=0.006, respectively) (Table II). It was also examined if the ratio of expression levels of DAP3 and DELE1 may be of interest but no significant difference between tumour and normal tissues was identified (Table II). DAP3 and DELE1 were respectively

Table II. Transcript levels of DAP3 and DELE1 in colorectal cancers.

		DAP3/GAPDH		DELE1/GAPDH		DAP3/DELE1 ratio		DELE1/DAP3 ratio	
Factor	n=174	Mean ± SD	P-value[a]	Mean ± SD	P-value	Mean ± SD	P-value	Mean ± SD	P-value
Tissue type									
Normal	80	852±282	0.0073	816±223	0.0007	671638±383780		33.6±15.6	
Tumour	94	60.4±55.5		23±18.9		1743780±1379284	0.46	219388±219121	0.32
Paired normal	68	614±217	0.0065	737±230	0.0022	800594±456831		36.1±18.3	
Paired tumour	68	2.46±1.61		2.41±1.82		2285996±1937511	0.46	368±345	0.34
Differentiation									
High	2	0.0862±0.0495	0.81[b]	0.0317±0.0317	0.124[b]	3463±3461	0.723[b]	0.234±0.234	0.151[b]
Moderate	54	2.17±1.76	0.24 vs. High	1.289±0.793	0.12 vs. High	482620±328897	0.15 vs. High	30.5±24.2	0.22 vs. High
Poor	14	0.1237±0.0464	0.62 vs. High	133±133	0.34 vs. High	223±152	0.52 vs. High	1424310±1424301	0.34 vs. High
Node involvement									
Negative	39	0.1145±0.0378	0.25	0.809±0.689		15307±11904		42.4±32.4	
Positive	31	3.72±3.09		58.9±57.5	0.32	869584±601742	0.17	687596±687594	0.33
TNM staging							0.257[b]	12.7±10.5	0.617[b]
TNM1	9	0.0673±0.0305	0.257[b]	2.93±2.91	0.66[b]	6097±5283			
TNM2	30	0.1292±0.0486	0.29 vs. TNM1	0.1517±0.0599	0.37 vs. TNM1	18268±15674	0.47 vs TNM1	51.6±42.3	0.38 vs TNM1
TNM3	26	4.24±3.73	0.27 vs. TNM1	69.3±69 .	0.35 vs. TNM1	1076586±741109	0.16 vs. TNM1	830844±830842	0.33 vs. TNM1
TNM4	6	10.37±9.19	0.31 vs. TNM1	24.9±19	0.31 vs. TNM1	146±145	0.29 vs. TNM1	4.16±2.5	0.45 vs. TNM1
T-stage									
T1	2	0.01887±0.00883	0.443[b]	0.025±0.0232	0.411[b]	3.1±2.52	0.544[b]	0.958±0.78	0.408[b]
T2	10	0.0584±0.0285	0.22 vs. T1	2.63±2.62 .	0.35 vs. T1	6103±5282	0.28 vs. T1	11.29±9.49 .	0.31 vs. T1
T3	40	0.493±0.254	0.07 vs. T1	1.111±0.857	0.21 vs. T1	613039±425466	0.16 vs. T1	39.5±32.4	0.24 vs. T1
T4	18	5.47±5.27	0.32 vs. T1	102±102	0.33 vs. T1	29212±29134	0.33 vs. T1	1172957±1172954	0.33 vs. T1
Dukes staging									
A	7	0.0808±0.0382	0.222[b]	3.76±3.75	0.49[b]	7836±6751	0.69[b]	16±13.4	0.529[b]
B	33	0.1252±0.0444	0.46 vs. Dukes-A	0.139±0.0547	0.37 vs. Dukes-A	3813543±3796518	0.32 vs. Dukes-A	46.9±38.4	0.45 vs. Dukes-A

Table II. Continued.

Factor	n=174	DAP3/GAPDH		DELE1/GAPDH		DAP3/DELE1 ratio		DELE1/DAP3 ratio	
		Mean ± SD	P-value[a]	Mean ± SD	P-value	Mean ± SD	P-value	Mean ± SD	P-value
C	32	5.47±3.46	0.13 vs. Dukes-A	60.7±55.6	0.32 vs. Dukes-A	837377±579922	0.16 vs. Dukes-A	664676±664674	0.33 vs. Dukes-A
Anatomical locations									
Left colon	22	0.517±0.364		83.5±82.1		381033±352801		907399±906325	
Right colon	28	3.32±3.2		0.0642±0.0445		868459±601806		46.9±43.9	
Transverse colon	2	0.566±0.566		0.054±0.054		5.25±5.22		19.5±19.4	
Degree of invasion									
Rectum	22	0.418±0.294		1.98±1.58		6629901±6534644		7.23±5.36	
Non-invasive tumours	50	2±1.87		35.8±35.2		3054729±2621118		415920±415411	
Invasive tumours	26	0.717±0.379	0.50	1.6±1.33	0.34	520278±501286	0.43	3.77±1.88	
DFS									
Disease free	35	0.088±0.0222		0.0661±0.0372		3569682±3566832		680±640	
With cancer related incidences	23	4.71±4.47	0.31	79.9±78.4	0.32	696353±667844	0.39	997017±997011	0.33
Patients remaining well	36	0.0961±0.0228		0.0642±0.0361		3888757±3571007		680±640	
Death									
Patients died of colorectal cancer	22	4.7±4.48	0.32	83.7±82.1	0.32	694060±667980	0.26	997017±997011	0.33
Metastatic diseases									
No metastatic diseases	50	0.277±0.161		0.61±0.535		2862940±2457943		480±448	
With distant metastasis	19	6.15±5.58	0.31	98.1±95.8	0.32	34347±31121	0.3	1246265±1246264	0.33
Local recurrence									
With no local recurrence	58	1.86±1.6		31.5±30.8		2440911±2264820		356504±356068	
With local recurrence	7	0.363±0.17	0.36	0.0157±0.0154	0.31	81060±71508	0.07	0.017±0.0157	0.32

[a]By student t test with Bonferroni correction unless otherwise stated; [b]groupwise comparison by ANOVA test.

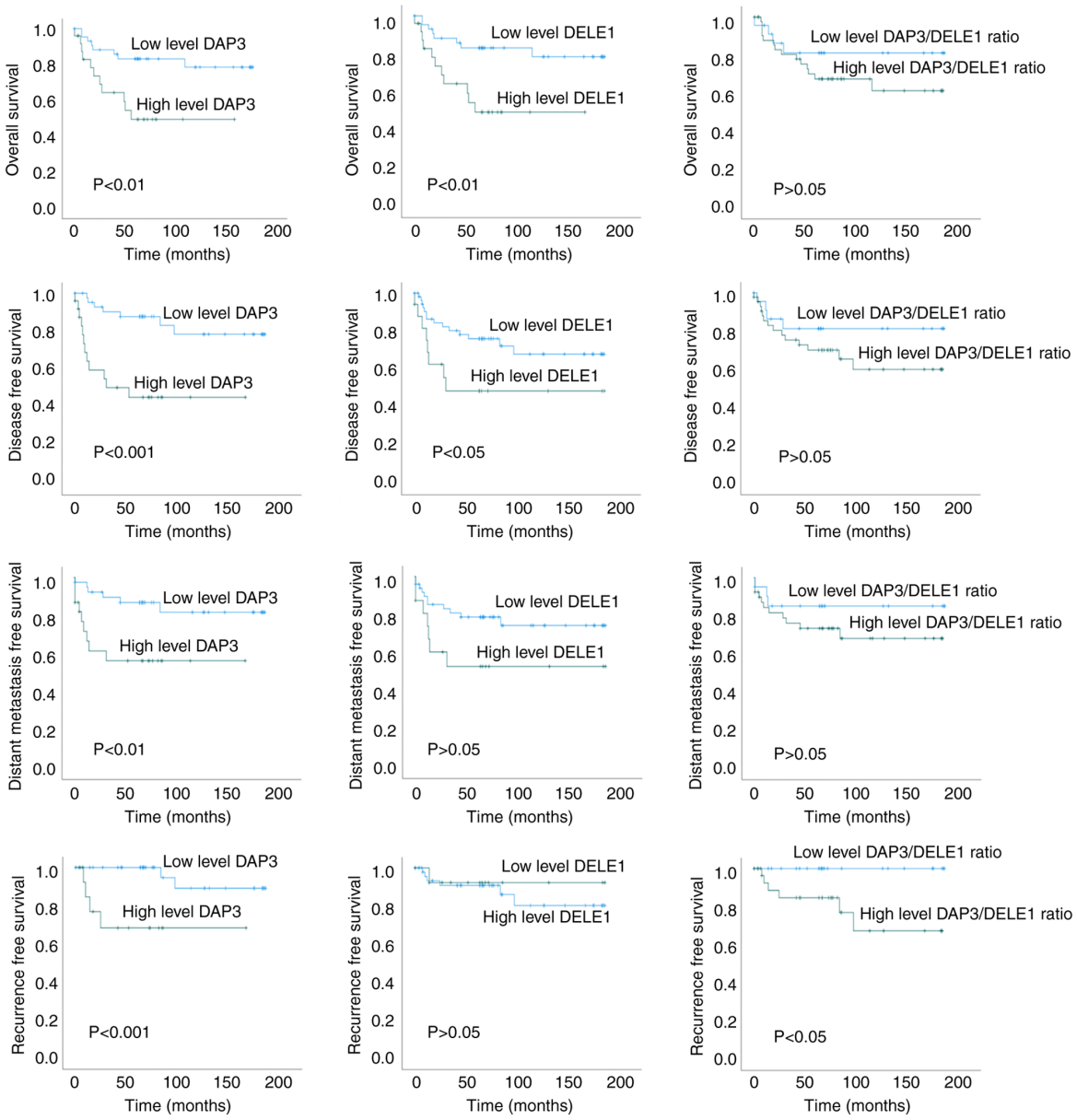


Figure 2. Kaplan-Meier survival analyses showed correlations between overall survival, disease-free survival, distant metastasis-free survival, recurrence-free survival and altered expression of DAP3, DELE1 and DAP3/DELE1 ratio. P-values shown are from the log ranked test. DAP3, death associated protein-3; DELE1, DAP3 binding cell death enhancer-1.

examined in the subgroups of different clinical and pathological groups including tumour staging (TNM staging and Dukes staging), differentiation, nodal status and invasiveness of tumour. The differentiation status of tumours did not show a consistent pattern for DAP3 (P=0.81) and DELE1 (P=0.124). Although tumours with positive node showed higher levels of DAP3 and DELE1, compared with node negative tumours, the differences were not significant (P=0.25 for DAP3 and P=0.32 for DELE1). There appeared to be a step wise increase of DAP3 from TNM1 to TNM4 tumours, from T1 to T3 stage tumours and from Dukes-A to Dukes-C tumours, though the differences were not significant (P=0.257 for TNM staging, P=0.443 for T staging and P=0.222 for Dukes staging). A similar observation was revealed with DELE1 expression in these groups (Table II). The expression levels of DAP3 and DELE1 in patients with different clinical outcome were also compared. As shown in Table II, tumours from patients who developed colon cancer-related incidence, died of colon cancer and developed distant metastasis tend to have higher levels of DAP3 than their counterpart group, yet the differences were not significant (P=0.31, 0.32 and 0.31 respectively). The same trend was observed with DELE1. The lack of statistical significance is largely owing to relatively smaller numbers in each group and the type of comparison, which further led to subsequent survival analyses by taking into consideration the survival time.

DAP3 and DELE1 are prognostic indicators for the OS and DFS and a good indicator for recurrence of CRC. DAP3 and DELE1 are strongly linked to the OS, DFS, distant metastasis free survival (DMFS) and recurrence of the patients (Fig. 2). Levels of DAP3 and DELE1 had significant association with

Table III. The value of DAP3, DELE1, and clinical factors in predicting the overall, disease free, metastasis free, and recurrence free survivals of the patients.

	Factors	Hazard ratio	P-value[a]
Overall survival	DAP3	3.427	0.01
	DELE1	2.227	0.076
	DAP3/DELE1 ratio	1.901	0.258
	Dukes stage	1.852	0.049
	TNM stage	1.243	0.043
	T staging	2.940	0.003
	Lymph node involvement	1.625	0.202
	Tumour differentiation	1.461	0.227
	Anatomical location	1.101	0.596
	DAP3	4.999	<0.001
Disease free survival	DELE1	2.388	0.05
	DAP3/DELE1 ratio	1.951	0.239
	Dukes Stage	2.366	0.010
	TNM stage	1.331	0.007
	T staging	2.464	0.008
	Lymph node involvement	1.625	0.037
	Tumour differentiation	1.259	0.622
	Anatomical location	1.103	0.558
	DAP3	3.691	0.014
Distant metastasis free survival	DELE1	2.434	0.067
	DAP3/DELE1 ratio	1.991	0.291
	Dukes Stage	2.864	0.007
	TNM stage	1.5	0.001
	T staging	4.705	0.001
	Lymph node involvement	1.736	0.03
	Tumour differentiation	1.521	0.399
	Anatomical location	1.543	0.252
	DAP3	6.326	0.038
Recurrence free survival	DELE1	0.613	0.651
	DAP3/DELE1 ratio	5.017	0.025
	Dukes Stage	2.191	0.169
	TNM stage	1.483	0.055
	T staging	17.525	0.008
	Lymph node involvement	1.742	0.193
	Tumour differentiation	1.167	0.853
	Anatomical location	1.037	0.915

[a]Multivariate analyses by Cox's Regression model. DAP3, death associated protein-3; DELE1, DAP3 binding cell death enhancer-1.

the DFS of the patients (P<0.001 and P=0.043, respectively, for DAP3 and DELE1) and the OS (P=0.006 and P=0.068, respectively, for DAP3 and DELE1) of the patients. Levels of both DAP3 and DELE1 were revealed to significantly differ between patients who developed recurrence from those who did not. Kaplan Meier's models showed a significant difference for both DAP3 and DELE1 in the time to recurrence.

Neither DAP3 nor DELE1 had differing levels in tumour with or without lymph node involvement. An interesting feature of the expression pattern of both DAP3 and DELE1 was a significant correlation of expression levels of both molecules in normal colorectal tissues (r=0.732, P<0.0001) but not in tumour tissues (P<0.05). Collectively, DAP3 (P=0.002) and DELE1 (P=0.01), together with T staging (P=0.024) are independent prognostic indicators for the OS of the patients. DAP3 (P=0.001), together with TNM staging (P=0.003), Dukes staging (P=0.026) and Nodal status (P=0.024) are also independent prognostic factors for the DFS of the patients. The significance and the hazard ratio of this relationship is summarized in Table III.

Association of DAP3 and DELE1 with drug resistance under clinical settings. Based on the TCGA dataset, it was found that patients who did not respond to chemotherapies had higher levels

Table IV. Response to the chemotherapeutic drugs with differential DAP3 and DELE1 expression.

Treatment	Responses	n	DELE1[a]	P-value[b]	DAP3[a]	P-value[b]
All chemotherapies	Responder	195	266 (42-517)	0.11	2340 (1337-5060)	0.74
	Non-responder	220	280 (134-618)		2388 (1102-5805)	
Bevacizumab	Responder	28	284 (169-494)	0.48	2405 (1383-4073)	0.041
	Non-responder	28	294 (199-486)		2658 (1979-4002)	
5-FU	Responder	148	260 (42-517)	0.36	2309 (1337-5060)	0.97
	Non-responder	155	279 (134-537)		2340 (1102-5306)	
Irinotecan	Responder	60	282 (42-517)	0.81	2303 (1350-4412)	0.98
	Non-responder	69	292 (162-537)		2268 (1102-5306)	
Oxaliplatin	Responder	97	260 (138-409)	0.14	2374 (1337-5060)	0.43
	Non-responder	77	279 (138-493)		2598 (1115-5805)	
Capecitabine	Responder	16	326 (151-485)	0.74	2284 (1486-3546)	0.81
	Non-responder	41	317 (164-618)		2326 (1103-4004)	

Dataset access was based on www.rocplot.com. [a]Median (interquartile range), [b]by Mann-Whitney U test, DELE1 expression was from 201977_s_at and DAP3 expression was from 208822_s_at. DAP3, death associated protein-3; DELE1, DAP3 binding cell death enhancer-1.

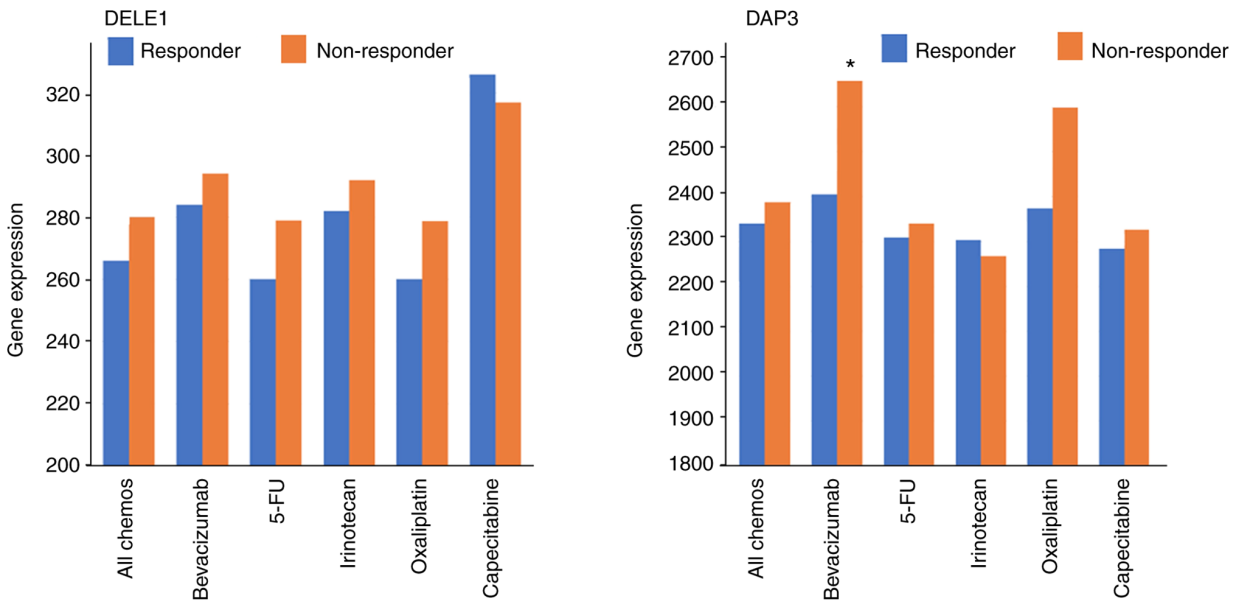


Figure 3. Response to chemotherapy with differential transcript DELE1 and DAP3 respectively. This was based on the public database (www.rocplot.com). Shown in the figure is the median of the respective group. *P<0.05 by Mann-Whitney U test. DAP3, death associated protein-3; DELE1, DAP3 binding cell death enhancer-1.

of DELE1 than those who responded, although this is yet to reach significance (P=0.11). The same but weaker trend was observed with DAP3. It appeared that high levels of DELE1 are exhibited in those who did not respond to 5-FU and Oxaliplatin and high levels of DAP3 in those who did not respond to Oxaliplatin (Fig. 3, Table III). There was no significant difference amongst the other groups except for in Bevacizumab, in which patients who had no response presented significantly higher DAP3 transcript than those who responded (P=0.041; Table IV).

Correlation between of DAP3 and angiogenesis markers in CRC. The finding that lower expression of DAP3 in the CRC tumour presented an improved response to the Bevacizumab led to the evaluation of its implication in the CRC by examining the TCGA-COAD cohort. Inverse correlation was revealed between DAP3 and most of the angiogenic markers/regulators, including Factor VIII (F8), CD34, platelet and endothelial cell adhesion molecule 1 (PECAM1), HIF1A, VEGFC, FGF1, FGF2, angiopoietin (ANGPT) 1, ANGPT2, SPHK1, Sphingosine-1-Phosphate Receptor (S1PR) 1, S1PR2 and S1PR3 (Fig. 4). Correlation between DAP3 and certain angiogenic factors was also analysed in the cohort using Spearman's correlation analysis. An inverse correlation was identified between DAP3 and PECAM1 or VEGFR3, but not others determined. By contrast, in this CRC cohort, DAP3 was positively correlated with VEGFR1 and VEGFR2 (Table V).

Table V. Correlation between DAP3 and angiogenic factors in the Beijing CRC cohort[a].

	VEGF A	VEGF B	VEGF C	VEGF D	VEGF R1	VEGF R2	VEGF R3	Podoplanin	PECAM1
DAP3	r=-0.122	r=-0.116	r=-0.08	r=-0.065	r=0.289	r=0.329	r=-0.272	r=-0.04	r=-0.292
	p=0.235	p=0.157	p=0.299	p=0.442	p<0.001	p=0	p=0.005	p=0.641	p<0.001
	n=97	n=151	n=170	n =143	n=161	n=152	n=106	n=137	n=143

[a]By Spearman's correlation test. Shown are correlation coefficients. DAP3, death associated protein-3; PECAM1, platelet and endothelial cell adhesion molecule 1.

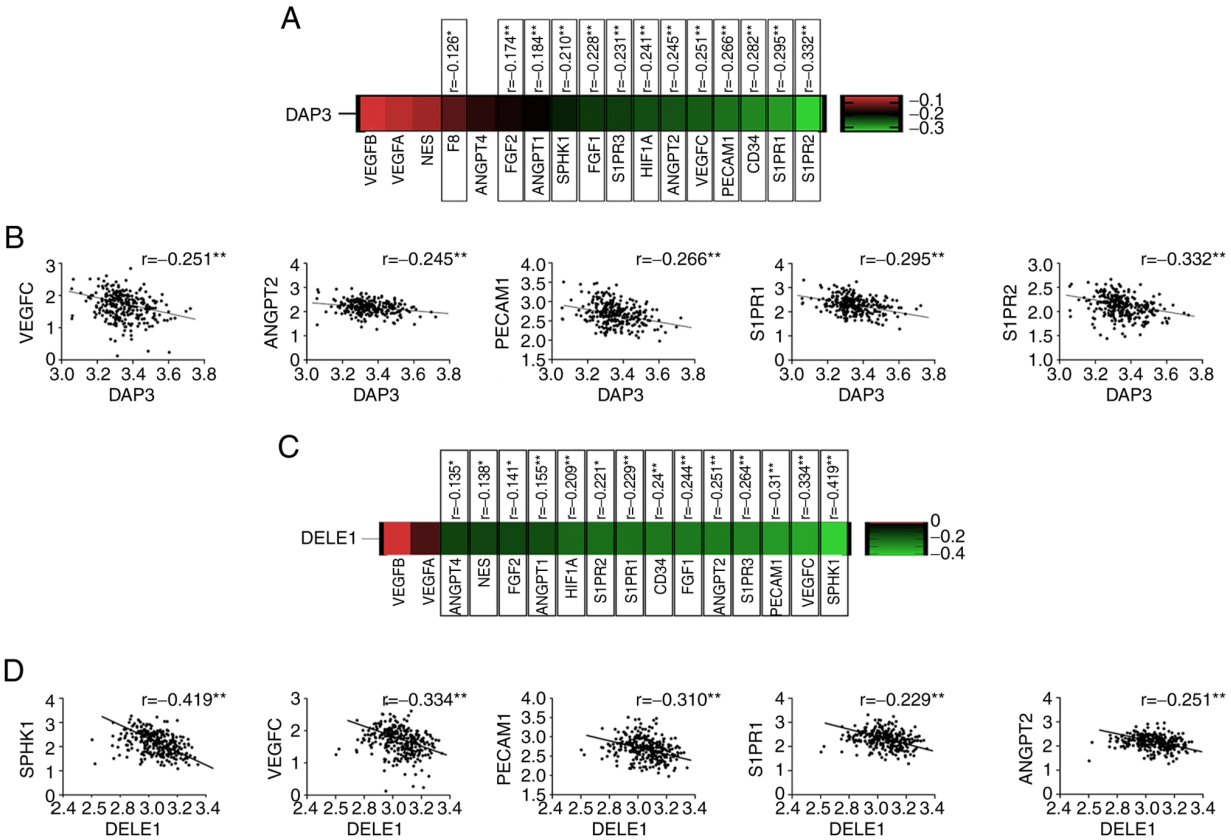


Figure 4. Correlation between DAP3 and DELE1 with angiogenesis biomarkers. (A and B) Correlation efficiency between DAP3 and angiogenesis markers in The Cancer Genome Atlas-COAD cohort was presented in (A) heatmap (A) and (B) scatter plots. (C and D) Association between DELE1 and angiogenesis biomarkers was revealed in (C) heatmap and (D) scatter plots. DAP3, death associated protein-3; DELE1, DAP3 binding cell death enhancer-1. **P<0.01.

Association of DAP3 and DELE1 with drug resistance under in vitro conditions. Using siRNA against DAP3 and DELE1, the expression of DAP3 and DELE1 in CRC cells was respectively knocked down (Fig. 5). Compared with the wild type colon cells, the control plasmid and control siRNA only resulted in minor and insignificant changes of the respective gene transcript (P>0.05) (Fig. 5, left panel for DAP3 and right panel for DELE1). The anti-DAP3 ribozyme transgene resulted in over 90% reduction of the DAP3 transcripts (P<0.001, vs. wild type cells and control transfection) (Fig. 5, left panel), whilst the anti-DELE1 siRNA resulted in over 50% reduction of the DELE1 transcripts (P<0.001 vs. wild type cells and control transfection). A double knockdown of DAP3 and DELE1 was also created in the same cells. Notably, DAP3 and DELE1 transcripts were even lower in double knockdown cells than the others (Fig. 5, left panel for DAP3 and right panel for DELE1).

With the sub-models of CRC cells created, the relationship of DAP3 and DELE1 expression levels and cancer cellular responses to chemotherapy drugs were then determined. As revealed in Fig. 6, knockdown of DAP3 and knockdown of DELE1 sensitised the cells to 5-FU, MTX and DTX. Notably, the DAP3/DELE1 double knockdown resulted in cells markedly sensitive to all the drugs tested, compared with single knockdowns and controls (Fig. 6).

DAP3 and DELE1 knockdown do not cause a significant change in mitochondrial metabolic rate. To evaluate if DAP3 knockdown, DELE1 knockdown and in particular the combined knockdown of both DAP3 and DELE1 had

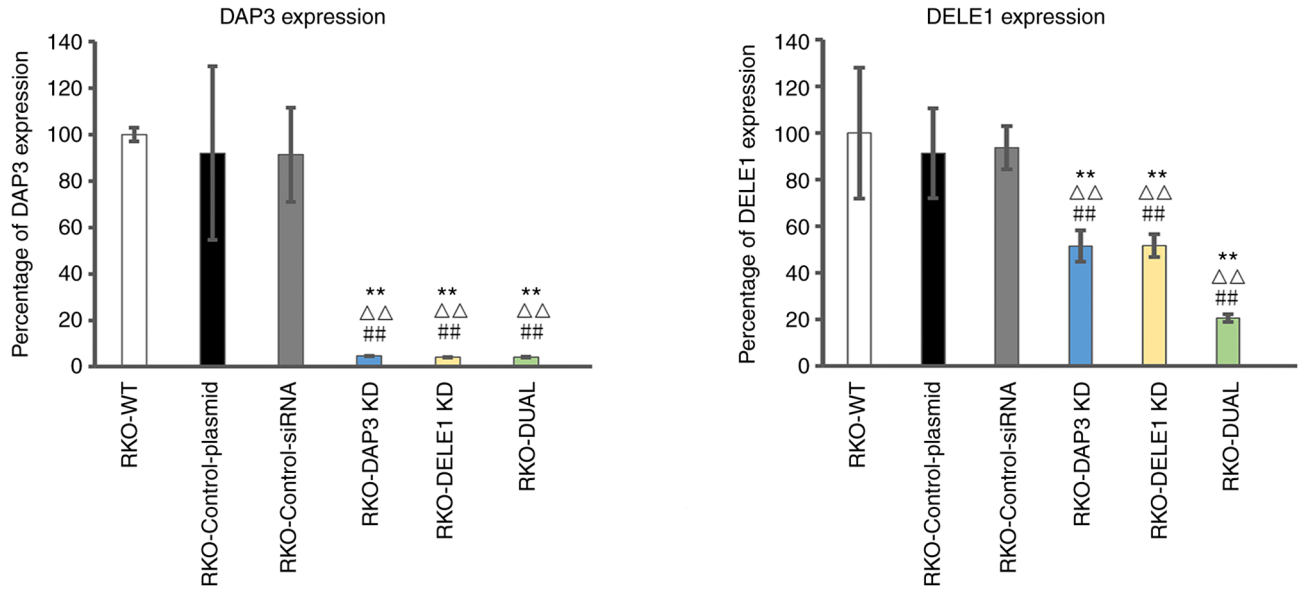


Figure 5. Validation of the DAP3 and DELE1 knockdown in RKO cells by quantitative PCR gene transcript analysis. Left panel: Expression of DAP3 in the cell models. The levels of expression in wild type, control transfection (control plasmid and control siRNA), DAP3 knockdown, DELE1 knockdown and DAP3/DELE1 dual knockdown cells were compared. Control transfections resulted in minor and statistically insignificant changes of DAP3 when compared with wild type cells (P>0.05) Right panel: Expression of DELE1 in the cell models. The levels of expression in wild type, control transfection (control plasmid and control siRNA), DELE1 knockdown and DAP3/DELE1 dual knockdown cells were compared. Control transfections resulted in minor and statistically insignificant changes of DELE1 when compared with wild type cells (P>0.05). Values presented in the figure are mean and standard deviation. Error bars represent standard deviation. **P<0.01 vs. RKO wild type cells, $^{\Delta\Delta}$P<0.001 vs. RKO-control-plasmid cells and $^{\#\#}$P<0.01 VS RKO-control-siRNA cells. DAP3, death associated protein-3; DELE1, DAP3 binding cell death enhancer-1; siRNA, small interfering RNA.

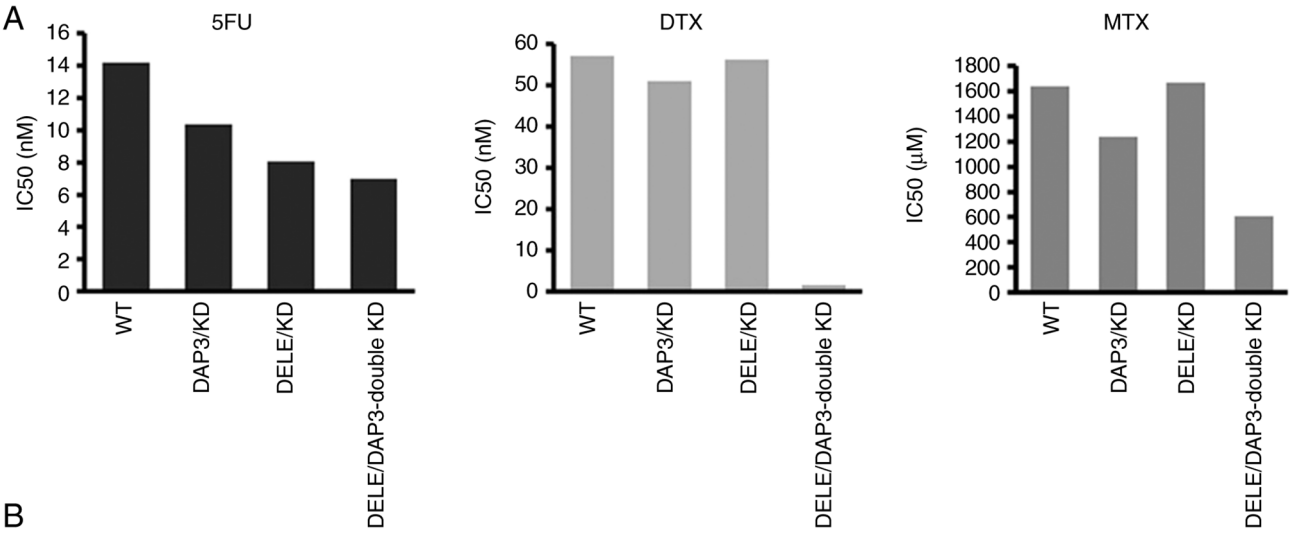
any impact on mitochondrial metabolism, the generated cells were examined for their rates of metabolism including glucose consumption, lactate production and reactive oxygen species (ROS). Elevated lactate production together with the enhanced cell growth rate were found in DAP3 knockdown and DAP3/DELE1 combined knockdown cells, compared with control cells. However, neither of the other pathways displayed a significant change after DAP3 knockdown, DELE1 knockdown or the DAP3/DELE1 double knockdown (Fig. 7).

Discussion

DAP3 is known as a critical molecule associated with apoptosis of various cell types in response to interferon gamma. Our previous analysis of its expression in a clinical cohort revealed that DAP3 was highly expressed in pancreatic tumour tissues and was significantly associated with shorter survival (6). A databased analysis has also shown that high levels of DAP3 in lung adenocarcinoma is linked to poor survival of the patients (28). However, DAP3 silencing promoted tumour progression including the enhanced adhesion, migration and invasion in breast cancer cells (29). These recent studies have thus indicated that the diverse and occasionally contrasting roles of DAP3 in different cell types and different tumour types are of significant interest including that observed in the current research (6,9,12,13,29). In the present study, expression of DAP3, on both protein (IHC) and gene transcripts levels, increased in colon cancer tissue compared with the normal adjacent tissue. DAP3 expression was correlated with the tumour staging again on both protein and gene levels. Higher DAP3 expression was associated with the poorer OS, DFS, DMFS and recurrence-free survival (RFS), according to the analysis in the clinical cohort. In order to determine the role of DAP3 in colon cells, *in vitro* DAP3 knockdown cell models were created using the ribozyme. Results from the cell toxicity test showed that downregulated DAP3 expression increased the cell sensitivity to the chemotherapeutic drugs. Additionally, differential DAP3 expression was observed in patients with different Bevacizumab responses in the current study. Since Bevacizumab exerted its activity as the inhibitor of the angiogenesis in the tumour, a panel of biomarkers were measured to investigate the correlation of DAP3 with neovascular in the TCGA-COAD dataset. From the current analysis, DAP3 expression was inversely correlated with most of the angiogenesis biomarkers, such as VEGFC, ANGPT2, PECAM1, S1PR1 and S1PR2, which indicated that high DAP3 expression may be associated with low angiogenic activities in the tumours and consequently affects response to the anti-angiogenic therapy. To date, there is no evidence showing a direct involvement of DAP3 in angiogenesis. Further exploration may shed light on how exactly DAP3 is involved in the tumour-associated angiogenesis in addition to its potential for predicting response to anti-angiogenic treatment.

Full length DELE1 was cleaved into shorter fragments (S-DELE1) in the cytosol to translate the mitochondria stress, and the stress signal was activated through an HRI dependent pathway which relayed the mitochondrial stress to ATF4. It has been shown that DELE1, via the OMA1-DELE1-HRI HRI mitochondrial pathway may mediate both detrimental and beneficial responses depending on the mitochondria stress sources (21). DELE1 was reported to act as an upstream molecule to activate Caspase-3, -8, and -9 to induce cell apoptosis. DELE1 silence suppressed caspase activation

A



B

	MTX (nM)	DTX (nM)	5FU (μM)
WT	1636.234	57.14	14.21
DAP3/KD	1238.77	51.014	10.38
DELE/KD	1666.34	56.15	8.06
DELE/DAP3-double KD	609.47	1.517	7.00

Figure 6. Cell toxicity to the chemotherapeutic drugs. (A) IC_{50} of the cell models in response to 5-FU, DTX and MTX. (B) IC_{50} table. 5-FU, 5-fluorouracil; DTX, Docetaxel; MTX, Methotrexate.

and enhanced viability (18). In the present study, markedly increased DELE1 expression was observed in the colon cancer tissues. Although no significant difference was shown between the early and the advanced stage from the IHC result, higher DELE1 was correlated with the significantly poor OS and DMFS, as demonstrated by our colon cancer cohort analysis.

Both DAP3 and its Binding Cell Death Enhancer-1, DELE1, were reported as the molecules inducing apoptosis, and the role of DAP3 was exerted via binding with DELE1 (18). Previous analysis in the colon cohort revealed that high level of DAP3/DELE1 ratio was associated with the poor RFS. To further investigate the interaction between DAP3 and DELE1, a dual knockdown cell model in RKO cells was created. While validating DAP3 knockdown efficiency, it was found that DELE1 expression was also downregulated simultaneously; in addition, in DELE1 silencing cells, low DAP3 expression was also observed, which indicated a positive regulation loop of these two proteins may exist in colon cancer cells. The recent findings that DAP3 acts as a variant splicing regulator of multiple genes provides some support to this possibility (17).

The findings that knocking down DELE1 and DAP3 influences drug sensitivity are interesting. *In vitro* cell toxicity tests demonstrated that DELE1 silencing in RKO cells enhanced the sensitivity of the chemotherapeutic drugs (5-FU and MTX). The resistance to the chemotherapeutic drugs was more significantly reduced in the dual knockdown group, compared with the other three groups. This is well supported by the clinical observations based on the TCGA dataset. Drug resistance has connections with mitochondrial metabolism as previously reported (30). It has been found that DELE1 protein contains a mitochondrial targeting sequence (18) and may act as a key player, together with OMA1, in mitochondria stress signalling pathways and indeed drug response in ovarian cancer cells (19-21,31) . It was therefore possible that DAP3 and DELE1 resulted drug resistance may have the mitochondrial link (32,33). For example, resistance to 5-FU has been indicated to increase mitochondrial mass, downregulate ATP synthase, and higher rates of oxygen consumption (34,35). Yet the exact links between mitochondrial function and drug resistance are less clear and need a great deal of investigation. ROS accumulation was considered to be a key ready for resistance to imatinib (36). A previous study revealed that resistance to paclitaxel is independent to glucose metabolism (37). In the present study, increased lactate production was found after the DAP3 silence compared with the control cells. Since lactate was involved in histone modification, namely, histone lysine lactylation, which is linked to the cancer progression and drug resistance (38), our finding on increased cell sensitivity to therapeutic drugs by DAP3 and DELE1 knock down indicated that DAP3 and DELE1-associated drug resistance in CRC is unlikely to be dependent on the glucose and ROS events and further investigation into other mitochondrial pathway and mitochondrial independent events is needed.

In conclusion, DAP3 and DELE1 are valuable prognostic indicators in human CRC. Since DAP3 and DELE1 may play a role in regulating mitochondrial stress (21), our findings suggested that targeting DAP3 and DELE1, at least in human CRC, represents a novel approach for improving current therapy.

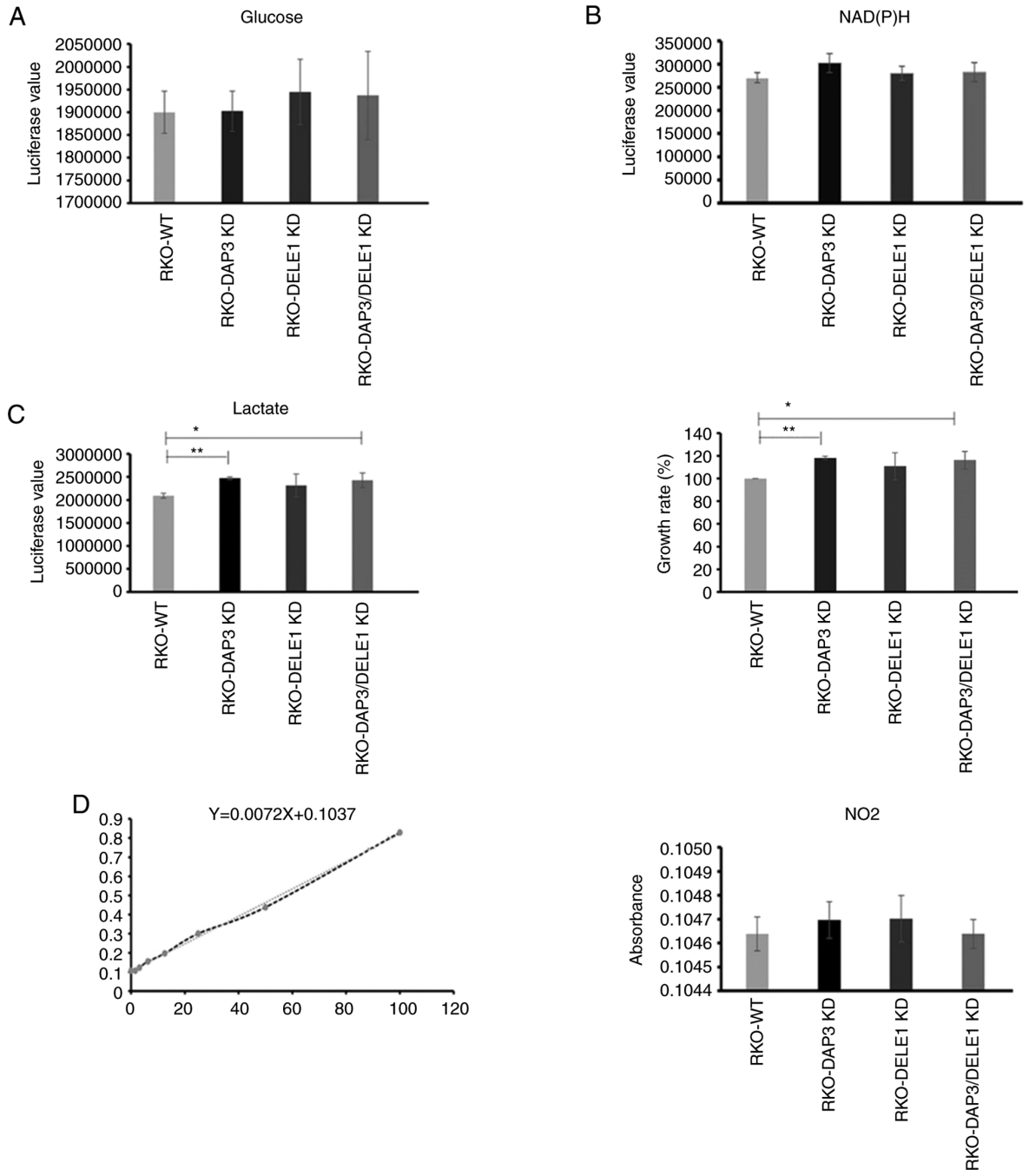


Figure 7. Metabolic of colorectal cancer cells following DAP3 and DELE1 knockdown. (A) Concentrations of extracellular glucose in different RKO cells were determined by luminescence measurement. (B) NAD(P)H measurements quantified by luminescence in different RKO cells. (C) Differential extracellular lactate expression and the ratio in different RKO cells. (D) NO_2 Standard curve and measurements of NO_2 in different RKO cells. Error bar stands standard error of means. *P<0.05 and **P<0.01 by Student's t-test. DAP3, death associated protein-3; DELE1, DAP3 binding cell death enhancer-1.

Acknowledgements

The authors wish to thank Dr Ann-Marie Toms of the University Hospital of Wales (Cardiff) for her assistance in patient's follow-up.

Funding

The present study was supported by Cardiff China Medical Scholarship, Taishan Scholars Project (grant no. ts20190991) and RealCan Fellowship.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WGJ, LY and AJS conceived the study. LS, JZ, HZ, LY and AJS conducted *in vitro* experiments. WGJ, RH and XS sourced the cohorts. AJS, LY, QPD and WGJ conducted genetic analysis. HZ, XS, AJ, JZ, TAM and FR conducted

histological and immunohistochemical analyses. AJS, WGJ, JZ and XS confirm the authenticity of all the raw data. All authors participated in data analyses and manuscript preparation. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was conducted in accordance with the Declaration of Helsinki and was approved by the local research ethics committee Bro Taff Research Ethics Committee (Ref. 05/DMD/3562) and by the Yuhuangding Hospital Research Ethics Committee (approval no. 2022-019; Yantai, China). Written informed consent was obtained from all subjects whose tissues were involved in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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