The pro-survival function of DLEC1 and its protection of cancer cells against 5-FU-induced apoptosis through up-regulation of BCL-XL

Guo-Hua Qiu · Wutang Que · Shanying Yan · Xintian Zheng · Xiaojin Xie · Cuiqin Huang · Xiaoyan Yang · Shing Chuan Hooi

Received: 14 March 2018 / Accepted: 15 September 2018 / Published online: 3 January 2019
© Springer Nature B.V. 2019

Abstract The tumor suppressor DLEC1 has been shown to promote cell proliferation when AP-2 α 2 is down-regulated in HCT116 stable clones, suggesting its pro-survival nature. However, the pro-survival function of DLEC1 has not been confirmed in other cells and its underlying mechanisms remain elusive. Therefore, we knocked down DLEC1 in a panel of cell lines and found that DLEC1 depletion caused various extents of cell death through intrinsic pathway. DLEC1 overexpression promoted cell survival and reduced cell death in cancer cells after 5-FU treatment, while DLEC1 down-regulation sensitized cancer cells to 5-FU. Further studies demonstrated that DLEC1 attenuated the increase in cleaved PARP, caspase-3 and caspase-7, the activity of caspase-9 and the diffusion of cytosolic cytochrome c from

mitochondria. Our data also showed that BCL-XL was up-regulated by DLEC1 in stable clones after 5-FU treatment. Altogether, these results indicated that DLEC1 protects cells against cell death induced by 5-FU through the attenuation of active proteins in caspase cascade and the up-regulation of BCL-XL. Therefore, DLEC1 can be a pro-survival protein under certain circumstances and a potential therapeutic target for increasing sensitivity of cancer cells to 5-FU.

Keywords DLEC1 · Cell lines · Pro-survival · Intrinsic apoptosis · 5-FU · BCL-XL

G.-H. Qiu · S. Yan · X. Zheng · C. Huang · X. Yang
Fujian Provincial Key Laboratory for the Prevention and Control of Animal Infectious Diseases and Biotechnology, Longyan University, Longyan 364012, Fujian, People's Republic of China

G.-H. Qiu · S. Yan · X. Zheng · C. Huang · X. Yang
Key Laboratory of Preventive Veterinary Medicine and Biotechnology, Fujian Province Universities, Longyan University, Longyan 364012, Fujian, People's Republic of China

G.-H. Qiu (✉) · S. Yan · X. Zheng · C. Huang · X. Yang
College of Life Sciences, Longyan University,
Longyan 364012, Fujian, People's Republic of China
e-mail: qgh@lyun.edu.cn

G.-H. Qiu · X. Xie · S. C. Hooi (✉)
Department of Physiology, Faculty of Medicine, National University of Singapore, Singapore 117597, Singapore
e-mail: phshsc@nus.edu.sg

W. Que
Orthopedics Department, Longyan First Hospital,
Longyan 364000, Fujian, People's Republic of China

Introduction

Apoptosis is an orderly and tightly regulated, evolutionarily conserved and genetically controlled natural process of programmed cell death (Wlodkowic et al. 2011). Two major apoptotic pathways, namely, extrinsic and intrinsic apoptosis signaling pathways, have been defined in cells responsive to apoptotic stimuli (Cotter 2009; Wlodkowic et al. 2011). The extrinsic pathway is triggered from outside the cell by the binding of pro-apoptotic ligands to death receptors on the cell surface, such as Fas receptors, tumor necrosis factors, and TRAIL receptor families, and is initiated by caspase-8 (Li and Yuan 2008; Wlodkowic et al. 2011). In contrast, the intrinsic apoptotic signaling induces cytochrome c release from mitochondria. Cytosolic cytochrome c initiates the formation of apoptosome complex, which consists of cytochrome c, caspase adaptor proteins such as Apaf-1, and caspases (Li and Yuan 2008; Ow et al. 2008). Apoptosome formation results in caspase activation and caspase-9 is the pre-dominant initiator caspase in this pathway. The activated initiator caspases-8 or -9 starts a proteolytic cascade by cleaving downstream effector caspases, such as caspase-3, -6, and -7 (Ow et al. 2008; Wlodkowic et al. 2011). Of these, caspase-3 is believed to be the main executioner caspase and its activation is essential for both the intrinsic and extrinsic apoptotic cell death (Li and Yuan 2008; Miura 2012).

The intrinsic apoptotic pathway is mainly regulated by the members of B cell leukemia/lymphoma 2 (BCL-2) family. Approximately 20 members have been identified and can be divided into pro-apoptotic and pro-survival proteins according to their functions (Brunelle and Letai 2009; Zinkel et al. 2006). The pro-apoptotic members of this family such as Bad, Bax, Bid, and Bim move from other cellular compartments to mitochondria and promote the permeabilization of the mitochondrial outer membrane, and the release of cytochrome c to initiate apoptotic cascade in response to apoptotic stimuli (Cotter 2009; Rolland and Conradt 2010). Conversely, the pro-survival members of this family, including BCL-2, BCL-XL, BCL-W, MCL-1 and A1, preserve mitochondrial integrity and prevent the release of cytochrome c in the presence of apoptotic stimuli (Brunelle and Letai 2009; Rolland and Conradt 2010; Zinkel et al. 2006). They can suppress the pro-apoptotic function of pro-apoptotic

family members through direct interaction of a series of heterodimerizations, resulting in the overall balance of these different proteins and ultimately governing cell fate (Cotter 2009; Hennessy 2016). Among them, BCL-XL (BCL2-like 1 or BCL2L1) is a major pro-survival protein member of the BCL-2 family. It is overexpressed in a variety of cancers and provides cancer cells with better protection than BCL-2 against apoptosis (Nita et al. 2000; Simonian et al. 1997). Consistently, ectopic overexpression of BCL-XL has been shown to promote cell growth and inhibit cell death in cancer cells (Chen et al. 2009; Nita et al. 2000), whereas down-regulation of BCL-XL sensitizes cancer cells to several chemotherapeutic agents, such as 5-FU (Nita et al. 2000; Simonian et al. 1997; Vilenchik et al. 2002; Zhu et al. 2005).

The antimetabolite drug 5-FU eliminates cancer cells by inhibiting DNA and RNA synthesis and eventually activates apoptosis triggered by DNA damage (Longley et al. 2003; Wyatt and Wilson 2009). Therefore, it is widely employed as a chemotherapeutic agent for a variety of solid tumors, such as cancers of the gastrointestinal tract, head and neck, breast and ovary (Ghiringhelli and Apetoh 2015). In the past more than two decades, increased understanding of the mechanism of 5-FU action has led to the development of strategies to increase the anticancer activity of 5-FU. Despite these advances, drug resistance remains a significant limitation to the clinical application of 5-FU (Ghiringhelli and Apetoh 2015). Therefore, substantial research effort is still needed to improve the sensitivity of cancer cells in order to enhance the patients' response rate to 5-FU-based chemotherapy (Ghiringhelli and Apetoh 2015; Longley et al. 2006). One of the options is to identify the molecules that regulate cancer cell death in response to chemotherapy (Longley et al. 2006).

On the other hand, *in vitro* and *in vivo* experiments have demonstrated that DLEC1 is a tumor suppressor silenced by DNA methylation in a range of common tumors, such as ovarian, hepatocellular, gastric, lung, head and neck, prostate and breast carcinomas (Kwong et al. 2006; Qiu et al. 2008; Seng et al. 2008; Seven et al. 2015; Ye et al. 2014; Ying et al. 2009; Zhang et al. 2015). DLEC1 methylation is associated with the AJCC staging of hepatocellular carcinoma (Qiu et al. 2008), prostate cancer progression (Zhang et al. 2015) and a higher recurrence rate in gastric cancer (Ye et al. 2014). Therefore, it could be a

non-invasive epigenetic biomarker for diagnosis of prostate carcinoma (Zhang et al. 2015) and a predictor for recurrence in Chinese patients with gastric cancer (Ye et al. 2014). Moreover, we recently found that DLEC1 suppresses cancer cell growth in the presence of AP-2 α 2 and stimulates cell proliferation in the down-regulation of AP-2 α 2 in DLEC1 overexpression stable clones of HTC116 (Qiu et al. 2015). This result suggests that DLEC1 has pro-survival functions apart from a tumor suppressor.

Here, we report that DLEC1 knockdown caused cell death through the intrinsic apoptotic pathway in a panel of cell lines. Our results showed that DLEC1 protected cancer cells from 5-FU-induced apoptosis by the attenuation of increase in caspase-3, -7 and -9 activity, and the inhibition of cytochrome c release through up-regulation of BCL-XL. Consistently, down-regulation of DLEC1 increased the sensitivity of cancer cells to 5-FU. Therefore, DLEC1 may be one of the molecules that control cancer cell death in response to 5-FU.

Materials and methods

Cell culture

The following cell lines were originated from the indicated sources and cultured in the indicated regular media as recommended in the ATCC (Manassas, VA, USA) website and prepared according to the manufacturer's instructions: cancer cell lines HCT116 (colon, McCoy's 5A), RKO (colon, DMEM), Hela (Cervix, DMEM), Chang Liver (Cervix, DMEM), HepG2 (liver, DMEM), A498 (kidney, DMEM), MCF-7 (breast, DMEM), RCC4 (kidney, DMEM), LS174T (colon, RPMI), and HCCM (liver, DMEM), normal cell lines HEK293 (kidney, DMEM) and pseudo-triploid 293T (kidney, DMEM). HCT116 pcDNA31, DLEC1-3 and DLEC1-7 are stable clones, established and characterized as described previously (Qiu et al. 2015). All media (Sigma-Aldrich Corporation, St. Louis, MO, USA) were supplemented with 10% of fetal bovine serum (Invitrogen Corporation, Carlsbad, CA, USA) and all cell lines were cultured at 37 °C in a humidified 5% CO₂ incubator.

Gene silencing by RNAi

Synthetic stealth siRNAs were purchased from Invitrogen. The sense sequences for siRNA duplex are as follows: siDLEC1-5: 5' UGU ACU GGC AGG UCA UUC CAG GAG C, siDLEC1-6, 5' AUA AUC AGC UGC UUC CUA GCC GUG G; siBCL-XL1: 5' GGA GAU GCA GGU AUU GGU G, and siBCL-XL2: 5' UGA CCA GAC ACU GAC CAU C (Zhu et al. 2005). Briefly, $0.2\text{--}0.5 \times 10^6$ cells were seeded into a 6-well plate 24 h before transfection. 20 μM of siRNA duplex and 3 μL of Lipofectamine RNAiMax Reagent (ThermoFisher Scientific) were each diluted in 250 μL of Opti-MEM Reduced Serum Medium (ThermoFisher Scientific, Waltham, MA, USA), incubated together at room temperature for 10 min and then added into each well. The medium was replaced with regular medium after 4 h. 24 h post-transfection, cells were treated with control or the indicated concentration of 5-FU for 24 h as indicated. 72 h post-transfection, cells were harvested for further experiments.

Effect of caspase inhibitors

Treatment of caspase-3 inhibitor Z-DEVD-FMK, caspase-8 inhibitor Z-IETD-FMK, or general inhibitor Z-VAD-FMK (BD Bioscience, Franklin Lakes, NJ, USA) was performed according to a previous report (Huang et al. 2012) with minor modifications. Briefly, $0.3\text{--}0.6 \times 10^6$ cells were seeded in a 6-well plate and transfected with DLEC1 siRNA or control siRNA (scramble, SCR). 24 h post-transfection, cells were treated with control or caspase inhibitors (40 μM each) for 1 h. 72 h post-transfection, cell death was assessed by flow cytometry.

Flow cytometry and cell proliferation assay

Experiments were performed as described previously (Qiu et al. 2008). Briefly, for cell cycle analysis, all cells were harvested and fixed with 70% ethanol overnight, washed with PBS twice and subsequently stained with 50 $\mu\text{g}/\text{mL}$ propidium iodide solution in PBS. Cells were filtered before flow cytometry (Epics Elite Esp, BD Bioscience) analysis. The cell numbers were analyzed using WinMDI (V2.8). To monitor cell proliferation, 1000 cells of stable cells were plated in 96-well plates overnight, treated with 5-FU for 24 h

and incubated at 37 °C for the indicated duration. The numbers of viable cells were determined by the standard MTT assay.

Transient overexpression of DLEC1

Transfection of cells was carried out according to a previous description (Qiu et al. 2004). Briefly, cells were seeded in a 6-well plate and cultured overnight. 1 µg of expression plasmid containing DLEC1 or GFP, or empty control plasmid pcDNA31, was transfected using Lipofectamine 2000 Transfection Reagent (Invitrogen), according to the manufacturer's recommendations. Cells were treated with the indicated concentration of 5-FU at 37 °C for 24 h at 24 h post-transfection, and then harvested for flow cytometry assay at 72 h post-transfection.

Extraction of protein and Western blotting

Harvested cells were suspended in cold RIPA (1 × PBS, 1% NP40, 0.5% sodium deoxycholate, and 0.1% SDS) and the suspension was sonicated. Equal amount (determined by the Bradford method) of protein (50–80 µg) are subjected to 10–12% SDS-PAGE and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). The blot was blocked with 1x PBS buffer containing 0.1% Tween 20 and 5% (w/v) nonfat milk powder at room temperature for 1 h. The membrane was probed with an indicated primary antibody, subsequently with a horseradish peroxidase-conjugated anti-rabbit or an anti-mouse secondary antibody for 1 h. The immunoreactions were detected with the enhanced chemiluminescence substrate Super-Signal West Dura (Pierce, Thermo Fisher Scientific). Expression of GAPDH, β-tubulin or β-actin was used as a loading control. Primary antibodies were anti-DLEC1 (Sigma-Aldrich), anti-PARP, anti-caspase 3, anti-caspase 7, anti-BAX, anti-BCL-2, anti-BCL-XL (Cell Signaling Technology, Danvers, MA, USA), anti-β tubulin, anti-β actin and anti-GAPDH (Santa Cruz Biotechnologies Inc., Santa Cruz, CA, USA).

Determination of caspase 9 activity

Enzymatic activity of caspase 9 was determined using Caspase-Glo® 9 kit (Promega, Madison, WI, USA) according to the manufacturer's instruction. Cells

were seeded in a 96-well plate and cultured overnight before treatment with 5-FU for 24 h. The cells were lysed following the protocol provided. Luminescence of samples was measured in a plate-reading luminometer (TECAN) and the activities were calculated according to the manufacturer's recommendation.

Confocal immunofluorescence microscopic analysis

Cells were cultured on coverslips to 75% confluency. Apoptosis was induced by treatment with 10 µM 5-FU for 24 h. Cells were washed with PBS three times, fixed with 3.7% formaldehyde in PBS for 15 min, washed with PBS-S (PBS containing 0.1% saponin), and then incubated with 1% bovine serum albumin in PBS-S for 1 h. After six washes with PBS-S, cells were incubated with anti-cytochrome c antibody for 2 h at room temperature. After six washes with PBS-S, the coverslips were incubated with FITC-conjugated secondary antibodies (Sigma) for 1 h. After six washes with PBS-S, the coverslips were mounted upside down with anti-fade solution, sealed, and examined under a Zeiss microscope in the confocal mode.

Statistical analysis

The 2-tailed t-test was used to test the experimental differences for statistical significance. $p < 0.05$ was considered statistically significant.

Results

DLEC1 knockdown causes cell death in various cell lines

We have shown that DLEC1 may promote cancer cell proliferation in HCT116 DLEC1 stable clones (Qiu et al. 2015). In order to study the pro-survival role of DLEC1, we knocked down DLEC1 in a panel of cell lines. As shown in Table 1, DLEC1 knockdown led to the various extents of cell death, ranging from 8.4 to 66.4% in the cell lines tested. The percentages of cell death were found low in cancer cell lines HCT116, HCCM, RKO, and normal cell lines HEK293 and 293T from 7.6 to 15.6%, moderate in Hela, HepG2,

Table 1 Cell death by DLEC1 knockdown in various cell lines

Cell lines	UT	SCR	siD5	siD6
HCT116	3.2	3.5	10.5	11.6
HCC-M	5.6	6.9	10.7	11.8
RKO	2.3	2.2	8.4	12.1
HEK293	4.3	4.5	10.3	15.6
293T	4.1	3.2	10.5	12.8
Hela	6.9	8.9	22.3	26.4
HepG2	6.8	9.5	32.2	33.7
Chang liver	5.1	5.4	24.4	30.1
RCC4	2.1	1.8	36.5	38.8
A498	4.8	5.5	54.6	60.7
LS174	5.7	6.5	60.2	54.7
MCF-7	6.8	6.1	66.4	55.1

Cell lines were knocked down by DLEC1 siRNAs (siD5 and siD6), control (SCR) or left untreated (UT) and then subjected to flow cytometry analyses. The percentages of subG1 cells are shown with the data from a representative experiment, which has been confirmed with two additional experiments with similar results

Chang Liver and RCC4 from 22.3 to 38.8% and high in A498, LS174T and MCF-7 from 54.6 to 66.4%. Consistent with our previous finding that DLEC1 could stimulate cell growth, these results further suggest that DLEC1 has a pro-survival role and may be required for cell survival in at least some of cancer cell lines.

The cell death resulted from DLEC1 knockdown is mediated through intrinsic apoptotic pathway

To examine whether the cell death resulted from DLEC1 depletion in cell lines is apoptotic, we examined the effects of various selective caspase inhibitors on DLEC1 knockdown-stimulated cell loss. Therefore, apoptosis inhibitors z-VAD-fmk, z-DEVD-fmk and z-IETD-fmk were used to treat cancer cell line A498 after DLEC1 knockdown. The results showed that the cell death by DLEC1 knockdown was completely inhibited by z-VAD-fmk, partially by z-DEVD-fmk and none by z-IETD-fmk (Fig. 1). These data indicate that the cell death by DLEC1 knockdown is apoptotic and that caspase-8 is not involved. Therefore, the cell death caused by DLEC1 knockdown could be mediated not by the extrinsic, but by the intrinsic pathway since caspase-8 is an initiator caspase in the extrinsic pathway (Wlodkowic et al. 2011).

DLEC1 overexpression protects cells from death of 5-FU in stable clones

5-FU could trigger intrinsic apoptotic pathway through inducing DNA damage (Yang et al. 2009). Therefore, to study the role of DLEC1 in the intrinsic pathway, we first treated HCT116 DLEC1 stable clones (Qiu et al. 2015) with 5-FU. In these stable clones, DLEC1 expression level was higher in clone DLEC1-7 than in clone DLEC1-3 (Qiu et al. 2015). Under normal conditions, DLEC1 suppressed

Fig. 1 The cell death by DLEC1 knockdown is not inhibited by IETD in cancer cell line. Cancer cell line A498 was knocked down by DLEC1 siRNA (siDLEC1-5) and control (SCR) and then treated with apoptosis inhibitors VAD (pan inhibitor),

DEVD (caspase 3 inhibitor) and IETD (caspase-8 inhibitor). The percentages of subG1 cells are shown with the data from a representative experiment, which has been confirmed with two additional experiments with similar results

Fig. 2 DLEC1 overexpression protects cells from death of 5-FU in stable clones. **a** Time course of DLEC1 stable clones (DLEC1-3, DLEC1-7) with 5-FU treatment (5 μM) starting from the time at 5-FU addition. **b** Dose response of HCT116 stable clones DLEC1-3 and DLEC1-7 to 5-FU at day 3 after 5-FU treatment. (0, 5, 10, 20 and 40 μM). The absorbance values were measured at 540 nm using MTT assay and the relative absorbance was calculated by standardizing the absorbance to

that at day zero (**a**) or at 5-FU zero μM (**b**). **c** Percentage of cell death of HCT116 DLEC1 stable clones DLEC1-3 and DLEC1-7 induced by 5-FU treatment. (0, 5, 10 and 20 μM) **d** Percentage of cell death induced by 5-FU treatment (0, 5 and 10 μM) in transient overexpression of DLEC1 clone in HCT116. All data are presented as mean ± SE in triplicates and are one representative of at least two independent experiments. * $p < 0.05$; ** $p < 0.01$

cell growth, and the suppression of cell growth was proportional to DLEC1 expression levels (Fig. 2a, upper), in accordance with the tumor suppressor function of DLEC1. However, under the treatment with 5 μM of 5-FU, the cell numbers of DLEC1-3 and -7 were vigorously increased till day 4 with more cells in DLEC1-7 than in DLEC1-3, whereas the cell numbers of vector control were only slightly increased till day 3 (Fig. 2a, lower) from the time of 5-FU addition. This result suggests that DLEC1 stable clones can survive longer than vector control under 5-FU treatment. Furthermore, the numbers of surviving cells and the tolerated concentration of 5-FU

were detected positively proportional to DLEC1 expression level according to the dose responses of stable clones to 5-FU (Fig. 2b). On the other hand, less cell death was detected in DLEC1-3 and -7 with the least cell death in DLEC1-7 at various concentrations of 5-FU (Fig. 2c). Moreover, less cell death was also detected in HCT116 cells with transient overexpression of DLEC1, compared to vector and GFP controls after 5-FU treatment (Fig. 2d). Therefore, the above data indicate that DLEC1 overexpression protects cancer cells against apoptosis induced by 5-FU.

Fig. 3 DLEC1 knockdown sensitizes cells of cancer cell lines to death by 5-FU. Cell lines were knocked down by DLEC1 siRNAs (siDLEC1-5 and siDLEC1-6), control (SCR) or untreated (UT) and then subjected to flow cytometry analyses. **a** The percentage of cell death in HCT116 stable clone of DLEC1-7 after DLEC1 was knocked down and treated with 5-FU (0, 2, 5 and 10 μ M). **b** The full-length of DLEC1 protein levels in HCT116 stable cells of DLEC1-7 determined by

immunoblotting 72 h after knocking down by DLEC1 siRNAs (siDLEC1-5 and siDLEC1-6) or control (SCR). **c, d** Percentage of cell death in cell lines HepG2 (**c**) and 293T (**d**) initiated at 48 h after DLEC1 knockdown and 24 h after 5 μ M of 5-FU treatment. All data in **a, c** and **d** are presented as mean $\pm$ SE intriplicates and are one representative of at least two independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.01$

DLEC1 knockdown sensitizes cells to death of 5-FU in cancer cell lines

Given that DLEC1 overexpression renders cancer cells resistant to 5-FU, we studied the effects of DLEC1 knockdown on cell survival after 5-FU treatment. As shown in Fig. 3a, compared to SCR control, DLEC1 depletion increased 2- to > 4-fold of cell death in stable clones of DLEC1-7 after 5-FU treatment. Figure 3b shows the protein levels of DLEC1 by siDLEC1 s in DLEC1-7 stable cells. Similarly, DLEC1 knockdown promoted cell death in cancer cell line HepG2 (Fig. 3c) and normal cell line 293T (Fig. 3d) after 5-FU treatment. The above results suggest that DLEC1 knockdown enhanced the cell sensitivity to 5-FU.

DLEC1 attenuates the increase of cleaved PARP, caspase-3, -7, -9 and cytochrome c release

The intrinsic pathway involves the DNA damage-induced release of cytochrome c, resulting in the activation of caspase cascade (Yang et al. 2009).

Therefore, to further investigate the role of DLEC1 in the intrinsic pathway, we assessed the alteration of these proteins in the intrinsic pathway. Immunoblot analysis demonstrated that as expected, 5-FU treatment significantly up-regulated the active forms of PARP, caspase-3 and -7 as seen in pcDNA31 samples (Fig. 4a). However, compared to vector control, DLEC1 overexpression decreased the levels of cleaved PARP, caspase-3 and -7 induced by 5-FU treatment. Moreover, the decreased amount of these proteins was correlated with the amount of DLEC1 level (Fig. 4a). Consistently, DLEC1 knockdown stimulated the level of cleaved caspase-7 caused by 5-FU treatment in HepG2 (Fig. 4b). DLEC1 overexpression suppressed the increase of caspase-9 activity (Fig. 4c) and prevented the cytosolic diffusion of cytochrome c from mitochondria in the majority of DLEC1-7 cells (Fig. 4d) induced by 5-FU treatment. Together, these data indicate that DLEC1 overexpression suppressed the release of cytochrome c and subsequent activation of enzymes in caspase cascade.

Fig. 4 DLEC1 attenuates the increase of cleaved PARP, caspase-3, -7, -9 and cytochrome c release. Cell lines with over-expression (HCT116 DLEC1 stable clones) or down-regulation (HepG2 siRNA knockdown) of DLEC1 were treated with 5-FU (0, 5 and 10 μM) and then subjected to Western Blotting analyses. **a** The levels of cleaved PARP, caspase-3 and -7 in HCT116 stable clones (DLEC1-3 and DLEC1-7) after 5-FU treatment. **b** The level of cleaved caspase-7 in HepG2 after

DLEC1 knockdown by DLEC1 siRNAs (siDLEC1-5 and siDLEC1-6), control (SCR) or untreated (UT) and 5-FU treatment (0 and 10 μM). **c** The activity of caspase-9 in HCT116 DLEC1 stable clones (DLEC1-3 and DLEC1-7) after 5-FU treatment (0, 5 and 10 μM). **d** The subcellular localization of cytochrome c in HCT116 stable cells (DLEC1-7) after 5-FU treatment (0 and 10 μM). Scale bar represents 10 μm.

The protection of cells from 5-FU-induced apoptosis by DLEC1 is caused by the up-regulation of BCL-XL

The BCL-2 family of proteins plays an important role in regulating intrinsic apoptosis. Hence, we examined the involvement of BCL-2, BAX and BCL-XL proteins in 5-FU-treated stable cells. We found that compared to no change of BCL-2, BAX and BCL-XL proteins in control stable cells, DLEC1 overexpression did not alter BCL2, induced BAX slightly but up-regulated BCL-XL significantly and proportionally to both DLEC1 level and 5-FU concentration after 5-FU

treatment (Fig. 5a). To examine whether BCL-XL is involved in the protection of cell death by 5-FU, we knocked down BCL-XL in DLEC1 stable clone DLEC1-7 and found that cell death was increased after 5-FU treatment (Fig. 5b). Accordingly, cleaved PARP and caspase-7 were up-regulated after BCL-XL knockdown and 5-FU treatment (Fig. 5c). Thus, the above data indicate that the protection of cells by DLEC1 against apoptosis caused by 5-FU could be mediated at least partially through the up-regulation of BCL-XL.

In summary, we demonstrated that DLEC1 has a pro-survival function. Our results established that

Fig. 5 The protection of cells from 5-FU-induced apoptosis by DLEC1 is caused by up-regulation of BCL-XL. **a** The up-regulation of BCL-XL after 5-FU (0, 5 and 10 μM) treatment in HCT116 DLEC1 stable clone DLEC1-7. **b** The increase of cell death after BCL-XL knockdown and 5-FU (0, 5 and 10 μM) treatment in HCT116 DLEC1 stable clone DLEC1-7. **c** The induction of cleaved PARP and caspase-7 after 5-FU (0, 5 and 10 μM) treatment and BCL-XL knockdown in HCT116 DLEC1 stable clone DLEC1-7

DLEC1 protects cells against 5-FU-induced apoptosis by preventing the increase of cleaved PARP, caspase-3, -7, activity of caspase-9 and cytochrome c release, and by up-regulating BCL-XL. The lack of DLEC1 in cancer cells contributed to their increased sensitivity to 5-FU treatment.

Discussion

On the one hand, we and others have demonstrated that overexpression of tumor suppressor gene *DLEC1* is able to suppress cancer cell growth in vitro and

in vivo (Kwong et al. 2006; Qiu et al. 2008; Zhang et al. 2015). On the other hand, we have recently shown that DLEC1 may stimulate cancer cell proliferation in DLEC1 overexpression stable clones of HTC116 (Qiu et al. 2015). The current study further confirmed the pro-survival nature of DLEC1. Knocking down DLEC1 caused cell death in various cell lines, including normal cell lines (Table 1) through the intrinsic apoptotic pathway (Fig. 1), suggesting that DLEC1 is required for normal survival in at least some of the cell lines. Considering both the cell suppression and cell survival properties of DLEC1, we speculate that the exact amount of DLEC1 and/or the ratio between isoforms with various lengths are critical for the cell fate. When the amount of full-length DLEC1 is above some threshold, DLEC1 suppresses cell growth in the presence of normal AP-2α2 (Kwong et al. 2006; Qiu et al. 2008; Zhang et al. 2015) or promotes cell growth via the down-regulation of AP-2α2 (Qiu et al. 2015). When the amount of full-length DLEC1 is below that threshold, and/or the ratio between isoforms is changed, cells may not survive even with the normal level of AP-2α2 (present study). Therefore, DLEC1 appears to be involved in regulating both cell suppression and survival depending on the amount of full-length DLEC1 involved and/or the ratio between its isoforms. Similar functions of a gene product have been observed in several reports (Qiu et al. 2015). For example, dual regulation of cellular homeostasis by AP-2α have been proposed to account for the balance between cell survival and cell death through both inhibition and activation of apoptotic signaling pathways, possibly depending on the cell context (Muller et al. 2004), the specific isoforms of AP-2, the ratio between AP-2 isoforms and/or the ratio between isoforms of AP-2 and AP-2-modulating factors in cells (Orso et al. 2008). Moreover, as DLEC1 has several splice variants and isoforms (Daigo et al. 1999), c-FLIP has 13 distinct splice variants at mRNA level with 3 identified proteins: c-FLIP_S, c-FLIP_R, and c-FLIP_L (Lavrik and Krammer 2012). It is now clear that c-FLIP_S inhibits apoptosis through several pathways, and c-FLIP_L inhibits caspase-8 activation when expressed at high levels, but induces caspase-8 activation at low levels, although both pro-apoptotic and anti-apoptotic effects were initially suggested for c-FLIP (Longley et al. 2006; Safa 2012). Therefore, the functions of c-FLIP in regulating cell survival, proliferation, and carcinogenesis has been proposed

through various isoforms with distinct regulatory roles in these signaling pathways (Lavrik and Krammer 2012; Safa 2012). Similarly, the large protein of DLEC1 with 1755 amino acids includes shorter proteins with distinctive functions (Daigo et al. 1999). In the present study, besides the full-length DLEC1, siRNAs against DLEC1 can also knock down other shorter variants of DLEC1 and/or disrupt the ratio balance between various isoforms, and may thus cause cell death (Fig. 2). Furthermore, the cell death by DLEC1 siRNA appears to be dependent on the cell type, because we found that DLEC1 depletion led to varying degrees of cell death in cell lines from different types of cancer (Table 1). Even in the same type of colorectal cell lines, down-regulating c-FLIP enhances their sensitivity to cytotoxic drugs with differing mechanisms (Longley et al. 2006).

Our present study showed that while DLEC1 overexpression protected cells against apoptosis induced by 5-FU (Fig. 2), DLEC1 depletion increased cell death from 5-FU treatment in both cancer and normal cell lines (Fig. 3). In c-FLIP, overexpression of c-FLIP_L inhibited apoptosis of CRC cells in response to the chemotherapeutic agents, including 5-FU. However, c-FLIP_S overexpression failed to protect CRC cells from 5-FU-induced apoptosis, but instead somehow enhanced apoptosis in some cell lines (Longley et al. 2006). Similarly, the protective roles by DLEC1 against 5-FU may also differ among the various isoforms of DLEC1. Therefore, DLEC1, either its full length or shorter isoforms, may be one of the molecules that regulate cancer cell apoptosis in response to 5-FU and thus a potential target molecule with the therapeutic property of enhancing the sensitivity of cancer cells to 5-FU.

In summary, we demonstrated here that depletion of DLEC1 causes cell death through the intrinsic apoptotic pathway. DLEC1 protects cells against apoptosis induced by 5-FU through the attenuation of active caspases and cytochrome c release, and by up-regulation of BCL-XL. Down-regulation of DLEC1 sensitizes cancer cells to 5-FU treatment, revealing the possible new targets for therapeutic interventions. Nevertheless, many more investigations are needed to confirm the functions of various DLEC1 isoforms and define the molecular mechanisms by which DLEC1 and its isoforms contribute to cell suppression and cell survival.

Acknowledgements This study was supported by the Major Project of Fujian Provincial Science and Technology Department (2014NZ0002-3), Longyan Science and Technology Scheme (2017LY73), Natural Science Foundation of Fujian Province (2018J01456) and the National University of Singapore ARF Grants R-185-000-074-112 and R-185-000-087-112.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

References

- Brunelle JK, Letai A (2009) Control of mitochondrial apoptosis by the Bcl-2 family. *J Cell Sci* 122:437–441
- Chen S, Dai Y, Pei XY, Grant S (2009) Bim upregulation by histone deacetylase inhibitors mediates interactions with the Bcl-2 antagonist ABT-737: evidence for distinct roles for Bcl-2, Bcl-xL, and Mcl-1. *Mol Cell Biol* 29:6149–6169. <https://doi.org/10.1128/mcb.01481-08>
- Cotter TG (2009) Apoptosis and cancer: the genesis of a research field. *Nat Rev Cancer* 9:501–507
- Daigo Y, Nishiwaki T, Kawasoe T, Tamari M, Tsuchiya E, Nakamura Y (1999) Molecular cloning of a candidate tumor suppressor gene, DLC1, from chromosome 3p21.3. *Can Res* 59:1966–1972
- Ghiringhelli F, Apetoh L (2015) Enhancing the anticancer effects of 5-fluorouracil: current challenges and future perspectives. *Biomed J* 38:111–116. <https://doi.org/10.4103/2319-4170.130923>
- Hennessy EJ (2016) Selective inhibitors of Bcl-2 and Bcl-xL: balancing antitumor activity with on-target toxicity. *Bioorg Med Chem Lett* 26:2105–2114. <https://doi.org/10.1016/j.bmcl.2016.03.032>
- Huang BH, Zhuo JL, Leung CH, Lu GD, Liu JJ, Yap CT, Hooi SC (2012) PRAP1 is a novel executor of p53-dependent mechanisms in cell survival after DNA damage. *Cell Death Dis* 3:e442. <https://doi.org/10.1038/cddis.2012.180>
- Kwong J et al (2006) Candidate tumor-suppressor gene DLEC1 is frequently downregulated by promoter hypermethylation and histone hypoacetylation in human epithelial ovarian cancer. *Neoplasia* 8:268–278. <https://doi.org/10.1593/neo.05502>
- Lavrik IN, Krammer PH (2012) Regulation of CD95/Fas signaling at the DISC. *Cell Death Differ* 19:36–41. <https://doi.org/10.1038/cdd.2011.155>
- Li J, Yuan J (2008) Caspases in apoptosis and beyond. *Oncogene* 27:6194–6206. <https://doi.org/10.1038/onc.2008.297>
- Longley DB, Harkin DP, Johnston PG (2003) 5-fluorouracil: mechanisms of action and clinical strategies. *Nat Rev Cancer* 3:330–338. <https://doi.org/10.1038/nrc1074>
- Longley DB, Wilson TR, McEwan M, Allen WL, McDermott U, Galligan L, Johnston PG (2006) c-FLIP inhibits chemotherapy-induced colorectal cancer cell death. *Oncogene* 25:838–848. <https://doi.org/10.1038/sj.onc.1209122>

- Miura M (2012) Apoptotic and nonapoptotic caspase functions in animal development. *Cold Spring Harb Perspect Biol*. <https://doi.org/10.1101/cshperspect.a008664>
- Muller FU et al (2004) Transcription factor AP-2alpha triggers apoptosis in cardiac myocytes. *Cell Death Differ* 11:485–493. <https://doi.org/10.1038/sj.cdd.4401383>
- Nita ME et al (2000) Bcl-X(L) antisense sensitizes human colon cancer cell line to 5-fluorouracil Japanese journal of cancer research. *Gann* 91:825–832
- Orso F et al (2008) AP-2alpha and AP-2gamma regulate tumor progression via specific genetic programs. *FASEB J* 22:2702–2714. <https://doi.org/10.1096/fj.08-106492>
- Ow YP, Green DR, Hao Z, Mak TW (2008) Cytochrome c: functions beyond respiration. *Nat Rev Mol Cell Biol* 9:532–542. <https://doi.org/10.1038/nrm2434>
- Qiu GH et al (2004) The candidate tumor suppressor gene BLU, located at the commonly deleted region 3p21.3, is an E2F-regulated, stress-responsive gene and inactivated by both epigenetic and genetic mechanisms in nasopharyngeal carcinoma. *Oncogene* 23:4793–4806
- Qiu GH et al (2008) The tumor suppressor gene DLEC1 is frequently silenced by DNA methylation in hepatocellular carcinoma and induces G1 arrest in cell cycle. *J Hepatol* 48:433–441. <https://doi.org/10.1016/j.jhep.2007.11.015>
- Qiu GH, Xie X, Deng L, Hooi SC (2015) Tumor suppressor DLEC1 can stimulate the proliferation of cancer cells when AP-2a2 is down-regulated in HCT116. *Hepat Mon* 15:e29829. <https://doi.org/10.5812/hepatmon.29829>
- Rolland SG, Conradt B (2010) New role of the BCL2 family of proteins in the regulation of mitochondrial dynamics. *Curr Opin Cell Biol* 22:852–858. <https://doi.org/10.1016/j.ceb.2010.07.014>
- Safa AR (2012) c-FLIP, a master anti-apoptotic regulator. *Exp Oncol* 34:176–184
- Seng TJ et al (2008) DLEC1 and MLH1 promoter methylation are associated with poor prognosis in non-small cell lung carcinoma. *Br J Cancer* 99:375–382. <https://doi.org/10.1038/sj.bjc.6604452>
- Seven D, Yavuz E, Kilic E, Baltaci E, Karaman E, Ulutin T, Buyru N (2015) DLEC1 is not silenced solely by promoter methylation in head and neck squamous cell carcinoma. *Gene* 563:83–86. <https://doi.org/10.1016/j.gene.2015.03.004>
- Simonian PL, Grillot DA, Nunez G (1997) Bcl-2 and Bcl-XL can differentially block chemotherapy-induced cell death. *Blood* 90:1208–1216
- Vilenchik M, Raffo AJ, Benimetskaya L, Shames D, Stein CA (2002) Antisense RNA down-regulation of bcl-xL expression in prostate cancer cells leads to diminished rates of cellular proliferation and resistance to cytotoxic chemotherapeutic agents. *Can Res* 62:2175–2183
- Wlodkowic D, Telford W, Skommer J, Darzynkiewicz Z (2011) Apoptosis and beyond: cytometry in studies of programmed cell death. *Methods Cell Biol* 103:55–98. <https://doi.org/10.1016/b978-0-12-385493-3.00004-8>
- Wyatt MD, Wilson DM III (2009) Participation of DNA repair in the response to 5-fluorouracil. *Cell Mol Life Sci* 66:788–799. <https://doi.org/10.1007/s00018-008-8557-5>
- Yang SY, Sales KM, Fuller B, Seifalian AM, Winslet MC (2009) Apoptosis and colorectal cancer: implications for therapy. *Trends Mol Med* 15:225–233. <https://doi.org/10.1016/j.molmed.2009.03.003>
- Ye X, Feng G, Jiao N, Pu C, Zhao G, Sun G (2014) Methylation of DLEC1 promoter is a predictor for recurrence in Chinese patients with gastric cancer. *Dis Mark* 2014:804023. <https://doi.org/10.1155/2014/804023>
- Ying J et al (2009) DLEC1 is a functional 3p22.3 tumour suppressor silenced by promoter CpG methylation in colon and gastric cancers. *Br J Cancer* 100:663–669. <https://doi.org/10.1038/sj.bjc.6604888>
- Zhang L et al (2015) DLEC1, a 3p tumor suppressor, represses NF-kappaB signaling and is methylated in prostate cancer. *J Mol Med* 93:691–701. <https://doi.org/10.1007/s00109-015-1255-5>
- Zhu H et al (2005) Bcl-XL small interfering RNA suppresses the proliferation of 5-fluorouracil-resistant human colon cancer cells. *Mol Cancer Ther* 4:451–456. <https://doi.org/10.1158/1535-7163.mct-04-0162>
- Zinkel S, Gross A, Yang E (2006) BCL2 family in DNA damage and cell cycle control. *Cell Death Differ* 13:1351–1359. <https://doi.org/10.1038/sj.cdd.4401987>