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RESEARCH

Down-Regulation of BAX Gene During Carcinogenesis and Acquisition of Resistance to 5-FU in Colorectal Cancer

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Abstract Carcinogenesis and resistance to chemotherapy could be as results of expression variations in apoptosis regulating genes. Changes in the expression of apoptosis interfering genes may contribute to colorectal carcinogenesis and resistance to 5-Flourouracil (5-FU) during treatment schedule period. The present study aimed to evaluate the expression of pro-apoptotic and anti-apoptotic genes in colorectal cancer tumor tissues, normal adjacent tissues, and tumor colorectal cancer cell line during acquiring resistance to 5-FU in HT-29 based on Bolus treatment protocol. The normal and tumor tissues were obtained from hospital after surgery and total RNA was extracted for expression analysis. The HT-29 colorectal cancer cell line was cultured and exposed with 5-FU in three stages based on Bolus protocol. The MTT assay and Real Time PCR were carried out to determine the sensitivity to the drug and expression of desired genes, respectively. The obtained data showed that Proapoptotic genes, *BAX* and *BID*, were down-regulated in resistant derivate cells compared to wild type HT-29 cells. On the other hand Antiapoptotic genes, *CIAP1* and *XIAP*, showed upregulation in resistant cells compared to wild type ones. Furthermore, *BAX* and *FAS genes* showed down-regulation in tumor samples in comparison to normal adjacent tissues. In conclusion, the results of our study suggest that BAX down-regulation could contribute as an important factor during both colorectal carcinogenesis and cell resistance to 5-FU.

Keywords Colorectal cancer · Carcinogenesis · 5-FU · Chemoresistance · Apoptosis

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Introduction

Colorectal cancer (CRC) is the second common cause of cancer death worldwide, with incidence of 1 million new cases and mortality of more than 500,000 cases, annually [1]. New statistics indicate that the incidence of CRC in USA is decreasing, whereas in Asian countries the rate is rising which is due to westernized habits [2–4]. The evolution of colorectal cancer that arises from normal colonic mucosa and changes to early and late Adenomas and finally leads to invasive cancer, is associated with series of genetic and epigenetic events during the carcinogenesis [5]. The CRC carcinogenesis processes were grouped into LOH (Loss of Heterogeneity) and MSI (Microsatellite Instability) in which cells lost tumor suppressor genes and induce genetic instability in their genetic materials. Furthermore, alterations in different pathways such as Wnt, K-ras, TGF-beta and P53 pathways are suggested as possible reasons of CRC carcinogenesis [6]. The evasion of apoptosis was illustrated as an essential and mandatory feature for development of malignancies such as colorectal cancer [7].

Fluorouracil (5-FU) as an antimetabolite drug that irreversibly inhibits thymidylate synthase (TS) has been used as a backbone drug in combination with other pharmaceuticals in most regimens for treatment of metastatic colorectal cancer [8]. Thymine depletion following TS inhibition could causes thymineless stress and leads to mechanistically unknown cell death and apoptosis [9]. The 5-FU may be administered in different schedules such as a bolus intravenous [IV] injection weekly or daily for 5 days every 4 weeks, based on Mayo

Clinic Regimen [10, 11]. Chemo-resistance to widely-used anticancer drugs such as 5-FU is an important problem in treatment of cancers [12]. Accordingly, understanding the mechanisms by which cancerous cells become resistant to 5-FU is important step towards predicting and/or overcoming that resistance. Different mechanisms have been reported for acquiring resistance to 5-FU and apoptosis in human cancers [13], including the induction of thymidylate synthase in colon cancer cells [14], increased activity of deoxyuridine triphosphatase [15], methylation of the MLH1 gene in colorectal cancer cell lines [16], Down regulation of microRNA-34a, [17] induction of maternal embryonic leucine zipper kinase (MELK) [18], decreased expression of UMP kinase (UMPK) [19], over expression of *Bcl-XL* and *Bcl-2* genes in colon cancer cell lines [20] and Mcl-1in pancreatic cancer cell lines [21].

The efficacy of natural apoptosis and chemotherapy in killing cancer cells depends on the successful induction of apoptotic pathways, and defects in each part of apoptotic machinery could lead to decreased sensitivity or apoptosis resistance in neoplasia and chemotherapy [22]. Therefore, down-regulation or suppression of pro-apoptotic genes and/or up-regulation of anti-apoptotic genes might be involved in resistance to apoptosis in carcinogenesis and in chemoresistant cancerous cells.

In view of these, defects in apoptosis pathways could be counted as a mechanism for colorectal carcinogenesis and resistance to 5-FU drug; hence, we assumed that variations in expression of apoptosis modifier genes might lead to apoptosis resistance. The aim of this study was to investigate the role of pro-apoptotic and anti-apoptotic genes in the expression variations of nine pro-apoptotic and six anti-apoptotic genes in colorectal tumor and adjacent normal tissues as well as in HT-29 colorectal cancer cell line during in vitro acquiring resistance to 5-FU, based on bolus protocol; in order to determine the important genes in both processes.

Materials and Methods

Patient Specimens

Twenty five fresh frozen cancerous and adjacent normal specimens of colon and rectum tumors were provided by the IRAN NATIONAL TUMOR BANK which is funded by Cancer Institute of Tehran University. The normal non-tumor tissue was dissected at least 6 cm away from tumor site and confirmed by pathologist. The clinical data including Histology, Patient History and Family History of all samples were provided by Tumor Bank (Table 1). The inclusion criteria consist of sporadic colon and rectum mucinous and non-mucinous adenocarcinoma, no previous chemotherapy and no other prior cancers. The informed consents were obtained from all patients or close relatives by INTB staff.

Table 1 Clinical data of colorectal cancer samples

	Number of cases(percent)
Gender	
Male	14 (56 %)
Female	11 (44 %)
Age (years)	
<60	15 (60 %)
≥60	10 (40 %)
Site of primary	
Colon	13 (52 %)
Rectum	12 (48 %)
Histological type	
Non-mucinous adenocarcinoma	20 (80 %)
Mucinous adenocarcinoma	5 (5 %)
Tumor grade	
I	15 (60 %)
II	5 (20 %)
III	4 (16 %)
IV	1 (4 %)
T classification	
T2	4 (16 %)
T3	21 (84 %)

Cell Culture and 5-FU Exposure

The HT-29 colorectal adenocarcinoma cell line (ATCC HTB-38) was cultured in DMEM (Biosera, Sussex, UK) medium supplemented with 10 % heat-inactivated Fetal Bovine Serum (FBS), and 1 % Penicillin-Streptomycin (Life Technologies GmbH, Darmstadt, Germany) at 37 °C in a humidified atmosphere with 5 % CO2. The cells were seeded in 25 cm^2 flasks in equal concentration of about 1×10^4 cells per flask. 5-FU was purchased from Sigma-Aldrich and dissolved in Phosphate Buffered Saline (PBS) (Sigma-Aldrich, Hamburg, Germany), to make stock sample at concentration of 50 mM. Cell exposure to 5-FU was performed based on clinical clearance kinetics model (Bolus protocol) that was previously published by Pizzorno and Handschumacher [23]. Based on this protocol the cells expose to a 5-FU concentration that is similar to dose in which patients obtain in clinical regimens. In order to achieve this kinetic, a stepwise dilution of medium was carried out at definite time periods, after adding 5-FU. The cells exposed to 5-FU at concentrations of 500 μM, 250 μM, 100 μM, 20 μM, 2 μM and 0.5 μM for timed periods of 2 min, 15 min, 30 min, 1 h, 3 h and 24 h; respectively. This protocol was repeated successively for 5 days and consequently drug-free medium was added to allow cell recovery. The exposure protocol was repeated for three times after 3 weeks intervals that are corresponded to about 18 doubling time.

Cytotoxicity Assay by MTT

In order to assess acquiring resistance to 5-FU in wild and resistant HT-29 cell lines, the MTT cytotoxicity assay was carried out using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (Sigma, Hamburg, Germany). Firstly, cells were seeded at a density of 1×10^5cells/ml in a 24-well plate and incubated for 24 h at 37 °C and 5 % CO2. Afterward, Cells were treated with three different concentrations of 5-FU including 5 μM, 10 μM, and 25 μM for 24, 48, and 72 h. Then, MTT solution at 2 mg/ml was added for 4 h and then, acidic isopropanol was added to dissolve formazan, and absorbance of converted dye was measured at a wavelength of 570 nm (Sunrise, Tecan, Mainz-Kastel, Germany). The viability was defined as the absorbance ratio of treated cells to untreated cells.

RNA Extraction and cDNA Synthesis

The patient fresh-frozen samples were grinded in liquid nitrogen using mortar and pestle, and then transferred instantly into lysis buffer of extraction kit for following extraction. Furthermore, the total RNA from wild type and treated cell line were extracted 2 days after third exposure using AllPrep DNA/RNA Mini Kit (Qiagen, CA, USA). In the case of cell lines, in order to minimize variations in gene expression during trypsinization, the lysis buffer was poured directly on the cells in flasks. The quality and quantity of extracted RNA were measured via Agarose gel electrophoresis and Biophotometer (Eppendorf, Germany). The cDNA was synthesized using 500 ng of total RNA and PrimeScript RT Reagent Kit (TAKARA, Japan) regarding manufacture protocol.

Real Time PCR

The primer sequences for Real time PCR were extracted from Primer bank (http://pga.mgh.harvard.edu/primerbank/) (Table 2). The pro-apoptotic and anti-apoptotic gene expression variations between tumoral and adjacent normal tissues; and between wild type and resistant sample were measured by quantitative Real Time PCR in a Rotor-gene 6000 instrument (Corbett life science, Sydney, Australia) using SYBR Premix Ex Taq II (TAKARA, Japan). The total 20 μ reaction volume was contained 10 μ SYBR Premix, 1 μ cDNA, 1 μ of forward and reverse primers and 8 μ ddH_2O that was performed in two-step Real Time PCR (95 °C for 10 s and 60 °C for 35 s). Relative gene expression Changes between cDNA samples were determined with ΔΔCt method using Excel 2007 according to the formula; in which the GAPDH gene was used as internal control.

Statistical Analysis

The ΔΔCt were calculated using the standard formula and Microsoft Office Excel 2007. The statistical analysis was performed using SPSS 16.0 for Windows, and Student's *t* test was used for comparison of the gene expression values between two tumor and normal adjacent groups. Statistical significance was defined as $P<0.05$.

Results

MTT Assay

The effect of three different concentrations of 5-FU in three different time periods on growth inhibition of HT-29 wild type and resistant derivative cell lines was investigated, a dose and time-dependent growth inhibition in HT-29 cell lines was observed. The results showed moderate 5-FU resistance in derivative resistant cells compared to parental wild type (Fig. 1).

Real Time PCR Results

Wild Type Versus Resistant Cell Line

We found two anti-apoptotic genes, CIAP-2 and XIAP, were significantly upregulated and BCL-2 was significantly downregulated in resistant cells compared to wild type cell lines (Fig. 2). Furthermore, two pro-apoptotic genes BAX and BID showed significantly reduced expression in resistant cells (Fig. 3) (*P*.Value<0.05).

Tumor Versus Normal Sample

The normal/tumor quantitative gene expression ratio was calculated using SYBR Green dye and GAPDH gene as internal control. According to our data, two proapoptotic genes, BAX and FAS, were significantly down-regulated in tumor samples compared to samples (Fig. 4). Furthermore,, none of six anti-apoptotic genes showed significant variation in their expression between normal and tumor samples (Fig. 5) (*P*.Value<0.05).

Discussion

In this study, we evaluated the expression of a set of anti-apoptotic and pro-apoptotic genes in colorectal tumor samples, their normal adjacent tissues and in 5-FU resistant HT-29 cell line following bolus protocol exposure of parental cells to 5-FU. Generally, there are two inter-connected apoptotic pathways, including extrinsic and intrinsic mechanisms mediated by death receptors on the cell surface and mitochondria, respectively. Both pathways contain different elements that act in

Table 2 Real Time PCR primer sets: the primer sets for Real Time PCR were extracted from PrimerBank data base

Gene name	Primer sequence	Gene name	Primer sequence
DR4	F: ACCTTCAAGTTTGTCGTCGTC	BCL2	F: GCCTTCTTTGAGTTCGGTGG
	R: CCAAAGGGCTATGTTCCCATT		R: ATCTCCCGGTTGACGCTCT
DR5	F: GCCCCACAACAAAAGAGGTC	$BCLX_L$	F: GAGCTGGTGGTTGACTTTCTC
	R: AGGTCATTCCAGTGAGTGCTA		R: TCCATCTCCGATTCAGTCCCT
FAS	F: CACCCGGACCCAGAATACC	Survivin	F: AGGACCACCGCATCTCTACAT
	R: TGTTGCTGGTGAGTGTGCATT		R: AAGTCTGGCTCGTTCTCAGTG
CASP8	F: AGAGTCTGTGCCCAAATCAAC	XIAP	F: AATAGTGCCACGCAGTCTACA
	R: GCTGCTTCTCTCTTTGCTGAA		R: CAGATGGCCTGTCTAAGGCAA
BID	F: CCTTGCTCCGTGATGTCTTTC	CIAP1	F: TTTTCCCAGGTCCCTCGTATC
	R: GTAGGTGCGTAGGTTCTGGT		R: CCAATCTGACAAGATCGTGCT
BAD	F: CCCAGAGTTTGAGCCGAGTG	CIAP2	F: TTTCCGTGGCTCTTATTCAAACT
	R: CCCATCCCTTCGTCGTCCT		R: GCACAGTGGTAGGAACTTCTCAT
BAX	F: CCCGAGAGGTCTTTTTCCGAG	APAF1	F: AAGGTGGAGTACCACAGAGG
	R: CCAGCCCATGATGGTTCTGAT		R: TCCATGTATGGTGACCCATCC
BAK	F: ATGGTCACCTTACCTCTGCAA	GAPDH	F: AAGGTGAAGGTCGGAGTCAAC
	R: TCATAGCGTCGGTTGATGTCG		R: GGGGTCATTGATGGCAACAATA

cascades and consequently lead to apoptotic biochemical and morphological changes [24]. The cross-talk between the extrinsic and intrinsic pathways of apoptosis depends on proteolytic activation of pro-apoptotic Bid protein by caspase-8 [25]. Suppression in the expression of genes which are involved in initiation or progression of apoptosis, the pro-apoptotic genes, or over-expression in anti-apoptotic genes could result in apoptosis failure during carcinogenesis and/or generation of resistance to drugs.

In order to induce resistance to HT-29 colorectal cancer cell line, the bolus 5-FU treatment simulating clinical clearance kinetics was used as described previously [23]. In our study, the 5-FU treatments were carried out in DMEM normal media in which contains thymidine element, thus the drug could not affect on the cells via thymidine-less cell death induction as a primary mechanism of 5-FU action. Therefore it might be useful to conduct the treatments in the thymidine free media and compare the results with the normal conditions. After exposure to 5-FU, the derivative cells showed moderate resistance to 5-FU, as determined by MTT assay. The result showed moderate resistance of derivatives cells generated by Bolus protocol treatment compared to parental wild type HT-29 cell line in three different 5-FU concentrations after 24 h, 48 h, and 72 h (Fig. 1). The Real Time PCR results of Proapoptotic genes involved in initiation and progression of apoptosis showed that BAX and BID genes are significantly down-regulated in resistant derivative cells in comparison with HT-29 wild type cells.

Following the initiation of apoptosis, BAX protein undergoes a conformational change that leads to its insertion into outer membrane of mitochondria, forming voltage-dependent anion channel (VDAC) and oligomeric pores (MAC). Formation of VDAC and MAC result in permeabilization of mitochondrial outer membrane and release of Cytochrome C and other pro-apoptotic factors from mitochondria into cytosol; the processes that leads to activation of caspase

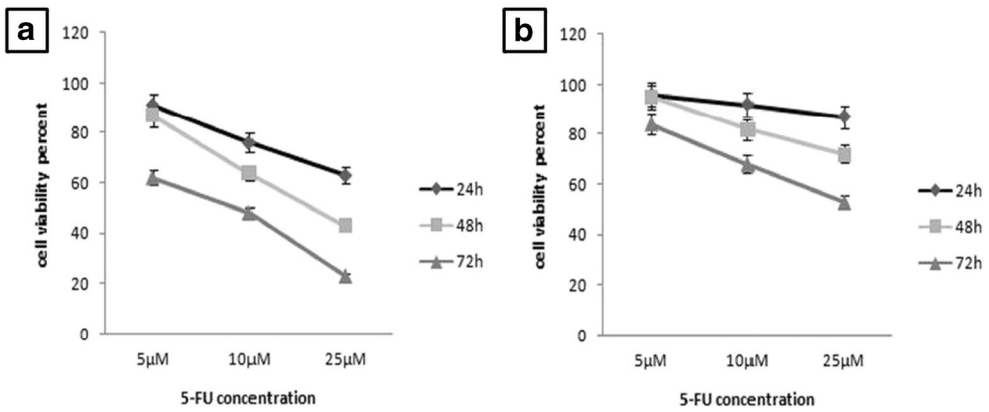

Fig. 1 MTT assay to measure cell viability in wild type HT-29 (**a**) and derivative 5-FU resistant HT-29 (**b**) after three different timings and concentrations of exposures to 5-FU: The results show moderate resistance of bolus-treated cell line derivatives (**b**) in comparison with wild type HT-29 cell line (**a**). The viability percent was defined as the absorbance ratio of treated cells to untreated cells × 100

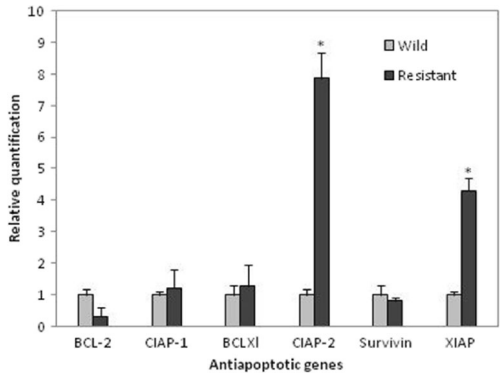

Fig. 2 Expression variation of anti-apoptotic genes between HT-29 wild type (*Gray*) and Bolus-induced resistant cell line (*Black*). CIAP-2 and XIAP genes show significant increase in expression level in resistant cells compared the wild type HT-29. The *asterisks* represent the significance of differences between the mean values of gene expressions ($p < 0.05$)

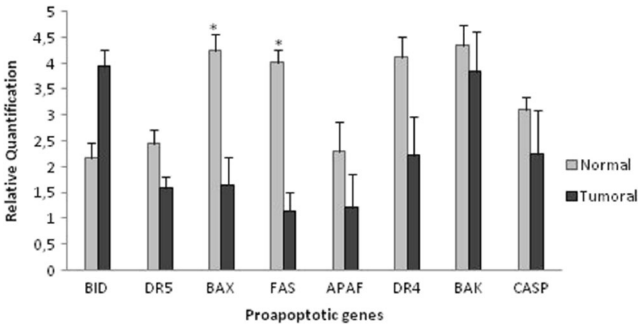

Fig 4 The expression variations of proapoptotic genes between tumor and normal adjacent tissues. The statistical analysis of data showed that BAX and FAS genes were significantly down-regulated in tumor samples (*black*) compared to normal adjacent tissues (*grey*). The *asterisks* represent the significance of differences between the mean values of gene expressions ($p < 0.05$)

enzymes [26]. Activation of caspase-8 followed by consequent BID cleavage and production of activated Bid (tBid), contributes to Cytochrome C mediated apoptosis. BID, a pro-apoptotic Bcl-2 protein containing the BH3 domain essential for Fas-mediated apoptosis, interacts with BAX and leads to insertion of BAX into mitochondrial outer membrane [27].

According to previous studies, Bax and the intrinsic apoptotic pathway are major effectors of 5-FU-induced apoptosis [28]. Up-regulation of the pro-apoptotic factors caspase-3, PARP and Bax as well as down-regulation of the Survivin have important roles in in-vitro chemo-sensitivity of 5-fluorouracil in colorectal cancer cell lines [29]. Bid protein as a pro-apoptotic Bcl-2 family of proteins is regulated by p53 and BID mRNA is increased by p53. In a previous study, it was demonstrated that compared to wild-type fibroblasts, BID-null mouse embryonic fibroblasts were more resistant to the DNA damaging agent adriamycin and the nucleotide analogue 5-fluorouracil [30]. In another study, it was shown that in comparison with Bid-insufficient HCC cell line Bid-abundant Hepatocellular carcinoma (HCC) cell line had a higher level of caspases activity

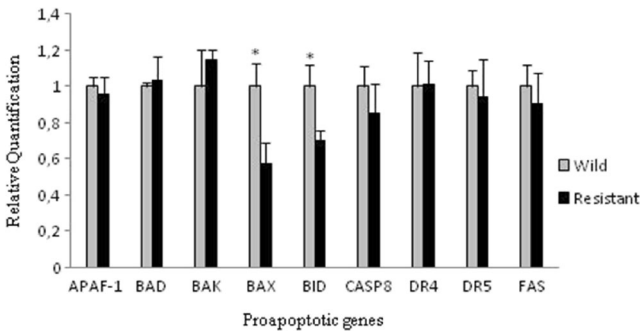

Fig 3 Expression variation of pro-apoptotic genes between HT-29 wild type (*Gray*) and Bolus-induced resistant cell line (*Black*). The data analysis showed that BAX and BID genes are down regulated in resistant cells (*black*) in comparison to normal wild-type cell line (*gray*). The *asterisks* represent the significance of differences between the mean values of gene expressions ($p < 0.05$)

induced by 5-fluorouracil (5-FU) and was more sensitive to drug-induced cytotoxicity [31].

In the present study, we found out that two IAP family anti-apoptotic genes, CIAP-2 and XIAP, were over-expressed in resistant cells compared to wild type sensitive cells (Fig. 2). The IAP family of proteins is considered as most important regulator of apoptosis due to their roles in regulation of both extrinsic and intrinsic pathways [26]. In previous studies, it has been confirmed that XIAP is one of the critical factors in the chemoresistance of pancreatic carcinoma cell, and its inhibition can increase the tumor sensitivity to 5-FU [32]. XIAP protein could bind and inactivate certain caspases such as caspases-9, 3, and 7; thereby, acting as an inhibitor of the effectors of apoptosis [33]. Besides, the role of cIAP2 in 5-FU chemoresistance was confirmed in a study on colorectal cancer cell lines and tissues. It was shown that the expression level of cIAP2 was increased in resistant cell lines and down-regulation of this gene led to more sensitivity to 5-FU [34].

The BAX/BCL2 ratio that declares a balance between proapoptotic and antiapoptotic genes has been mentioned as an important factor in clinical significance; in view of that the checkpoints are controlled by their expression levels. Our results show that the ratio is higher in resistant cell line

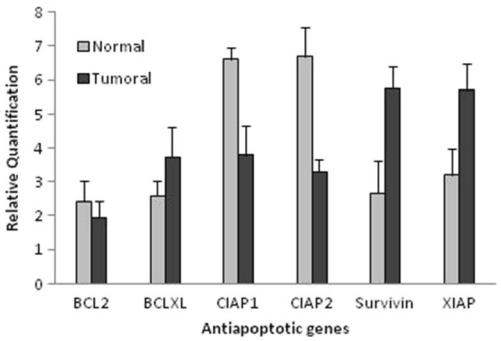

Fig. 5 The expression variations of antiapoptotic genes between tumor and normal adjacent tissues. The statistical analysis of data showed that there were no significant expression difference between tumor samples and normal adjacent tissue samples ($p < 0.05$)

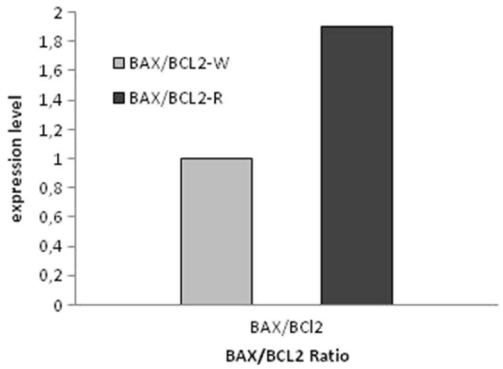

Fig. 6 The BAX/BCL2 ratios between HT-29 parental wild-type (*grey*) and derivatives resistant (*black*). The data shows that the ratio is higher in resistant cell line

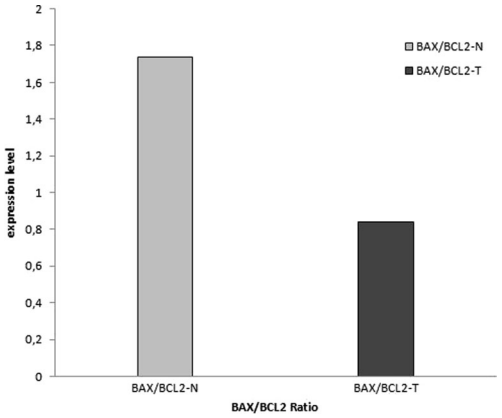

Fig. 7 The BAX/BCL2 ratios between normal (*grey*) and tumor (*black*) samples: The ratio is higher in normal samples in comparison to tumor samples

compared to the wild type sensitive cells (Fig. 6). This result declared that the higher BAX/BCL2 ratio indicates decreased sensitivity and induced resistance to 5-FU. The relative decrease in BCL2 expression in resistant cells is the reason of having higher BAX/BCL2 ratio in resistant cells. This result may show that decreased expression of Bax, not the Bax/Bcl-2 ratio, may play some roles in the relatively lower sensitivity of human HT-29 to 5-FU. In the previous study, it has been shown that patients who have higher BAX/BCL2 ratio do not benefit from 5-FU chemotherapy and those with low BAX/BCL2 ratio have better survival following treatment with 5-FU [35].

In another part of this study, the expression of Proapoptotic and Antiapoptotic genes were assessed among normal and tumor tissues samples. According to our data, the expression of two Proapoptotic genes, BAX and FAS, were significantly decreased in tumor samples. In addition, none of Antiapoptotic genes analyzed here showed significant variation in expression level between normal and tumor specimens (Fig. 4). FAS (CD95) receptor on the surface of the cells is important for triggering apoptosis through immune lymphocyte cells, and ignoring this death pathway mechanism leads to escape of tumor cells from immunosurveillance [24]. The mechanisms developed by tumor cells to overcome induction of Fas-FasL apoptosis in different cancers could be summarized as: increase in expression of Fas-associated phosphatase-1, decrease in expression of Fas on cell surface, lack of appropriate posttranslational modifications, and amplification of Fas decoy receptor. Furthermore, Fas deficiency in $Apc^{Min/+}$ mice leads to significant enhancement in colon tumors [36]. In our study, we showed that inappropriate expression of FAS gene that could let tumor cells escape from immune system, might has an important role in multi-step process of carcinogenesis. The present result is in accordance with previous studies that have indicated the FAS down-regulation during colorectal carcinogenesis [37, 38]. The decrease in FAS expression level might be as a result of promoter methylation as this gene has CpG islands in its 5′ promoter region. Furthermore, some mutations in transcription binding sites of FAS promoter may lead to its downregulation.

Bax protein has role in progression of apoptosis, and lack of functional Bax has been shown to induce resistance to apoptosis following treatment with chemotherapeutics [39]. Also, it has been noted that in Bax-deficient mice apoptosis is decreased, and induction of Bax expression in SCID mice leads to decrease in tumor growth as a result of re-sensitivity to apoptosis [24]. Furthermore, it has been shown that Bax expression is reduced from normal mucosa to primary tumor and to metastatic colorectal cancer suggesting that Bax down-regulation might be involved in metastasis progression [40].

In our study, Bax, a proapoptotic gene, has been significantly down-regulated in tumor samples. Since Bax is involved in downstream of P53 master gene, its reduced expression could impair the normal apoptotic cascade. Our findings support the previously mentioned notion that the reduction of Bax expression is an important factor during CRC carcinogenesis. The BAX/BCL2 ratio that is accounted as a relative ratio between Proapoptotic and Antiapoptotic proteins was determined by division of means for BAX expression to BCL2 expression levels. According to our analysis, the BAX/BCL2 ratio in normal tissues is higher than tumor tissue samples (Fig. 7), suggesting that this ratio could be used as a prognostic or predictive marker for colorectal cancer.

In conclusion, in this work, we showed that changes in expression of anti-apoptotic and Proapoptotic genes (e.g. BAX) are involved in colorectal carcinogenesis, and moderate resistance to 5-FU in HT-29 derivate based on Bolus protocol. Bax protein is a key regulator of apoptosis, induces apoptosis in colorectal epithelial cells, and lack or decrease in its expression could interfere with normal process of apoptosis in these cells, both during carcinogenesis or gaining resistance to chemotherapeutics.

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