

RESEARCH ARTICLE

Low Expression of the *bcl2* Gene in Gastric Adenocarcinomas in Mazandaran Province of Iran

Seyedeh Habibeh Mirmajidi², Ramin Ataee^{1*}, Ali Barzegar², Novin Nikbakhsh³, Mohammad Shaterpour¹

Abstract

Background: Gastric cancer accounts for about 8% of the total cancer cases and 10% of total cancer deaths worldwide. It is the second lethal cancer after esophageal cancer and is considered the fourth most common cancer in north and northwest Iran. The *bcl2* family has a key role in the regulation of apoptosis and change in its expression can contribute to cancer. This study initially scheduled to determine the expression of *bcl2* gene in tissue samples of adenocarcinoma cancer patients. **Materials and Methods:** A total of 10 samples of gastric adenocarcinoma and 10 of normal tissues from Sari hospital were selected and after DNA extraction from tissues, *bcl2* gene expression assayed by real-time PCR. **Results:** Our results demonstrated higher expression of the *bcl2* gene in control compared with cancer and marginal cancer tissues. **Conclusions:** On one hand *BCL2* plays an important role as an oncogene to inhibit apoptosis; on the other hand, it can initiate cell cycle arrest at G0 stage. Our observed association between its expression and patient survival is quite conflicting and may be tissue-specific. The data suggest expression both tumoural and non-tumoural(marginal) groups have lowered expression than controls ($P>0.05$). Due to the low number of samples we could not examine the relationship with clinicopathological features. However, *bcl-2* expression may be important for prognostic outcome or a useful target for therapeutic intervention.

Keywords: Gastric adenocarcinoma - *bcl2* gene - gene expression

Asian Pac J Cancer Prev, 16 (14), 6067-6071

Introduction

Gastric cancer is the fourth most common cancer in the world and the second most frequent cause of cancer deaths, accounting for 10.4% of cancer deaths worldwide (Parkin et al., 2002). There are 900000 new cases and 700000 gastric cancer-related deaths worldwide (Parkin et al., 2001). This cancer is more frequently affected men than women and the disease usually occurs over the age of 55 (Axon et al., 2006; Pinto et al., 1994). *H. pylori* infection is one of the main risk factors in 65–80% of gastric cancers. (Sung et al., 2007). Some kind of foods, such as smoked foods, salted fish and meat and pickled vegetables are associated with higher risk of gastric cancer. Also nitrates and nitrites in cured meats can be converted by certain bacteria as *H. pylori* into compounds that have been found to cause gastric cancer (WHO, 2004). Smoking increases the risk of developing gastric cancer significantly also some studies show increased risk of gastric cancer with alcohol consumption as well (WHO, 2004). In General, the various factors related to apoptosis such as bacteria, environmental, age, sex, genetic factors, the implemented

mutations in tumor's Suppressor genes and apoptosis related genes (Hopkins et al., 1998; Cho et al., 2005; Kim et al., 2005; Milne et al., 2009). Apoptosis is a natural process which causes the preserve of homeostasis in the natural tissues of the body by making a balance between the cellular proliferation and death, and removing the damaged and old cells. The control or any disorder in it has the effective role in malignant deformation process, cancer progress and metastases (Bold et al., 1997; Kamesaki et al., 1998). Apoptosis is done through two different pathways including the death-receptor pathway and mitochondrial pathway (Favaloro et al., 2012). The B-cell lymphoma 2 (*Bcl-2*) family represents apoptosis regulating proteins integrating diverse intra- and extracellularly generated survival and death signals. This protein family can be subdivided into proapoptotic members such as *BCL2*- associated X protein (Bax) and BAX-like death factor (Bak) and antiapoptotic/antiproliferative members like *Bcl-2* itself and *BCL2*-like 1 (*Bcl-xL*), protecting cells from apoptosis and inducing cell cycle arrest in G0 (Cory and Adams, 2002). *Bcl-2* gene, located at 18q21.3 and consists of three exons and two promoters (P1 and P2),

¹Pharmaceutical Sciences Research Center, Mazandaran University of Medical Sciences, ²Department of Basic Sciences, Sari Agricultural Sciences and Natural Resources University, Sari, ³Babol University of Medical Sciences. Babol, Iran *For correspondence: raminataee1349@gmail.com

which have different functions. The second promoter, P2, is located 1,400 bp upstream of the translation initiation site and functions as a negative regulatory element to the P1 promoter (Young and Korsmeyer, 1993; Seto et al., 1998). The *Bcl-2* gene codes for a 25KDa protein. The C terminal 21 aminoacids encode a stretch of hydrophobic aminoacids that are important in membrane docking: *Bcl-2* resides on the cytoplasmic face of the mitochondrial outer membrane, the nuclear envelope, and the endoplasmic reticulum (Antonsson et al., 2001). Assessment of apoptosis and individual components of the apoptosis pathway might therefore be relevant in determining the prognosis in an individual patient. There are two principal pathways leading to apoptosis, one begins in the plasma membrane with the presence of cell-surface death receptors and the other begins in the mitochondria. The mitochondrial pathway is regulated by members of the *Bcl-2* protein family (Cleland et al., 2010; Martinou and Youle, 2011).

Which of the dual functions' predominates seem to be tissue specific; for example, increased *Bcl-2* expression is associated with unfavorable outcome in B-cell chronic lymphocytic leukemia (CLL) and prostate cancer (Ofner et al., 1995; Zhang et al., 1998) whereas its expression is related to increased survival in colorectal and breast carcinoma (Keshgegian et al., 1998; Faderl et al., 2002). apoptosis markers, in particular *Bcl-2* and Bax expression, are receiving much attention for their relationship with the cellular response to genotoxic damage in experimental tumors (Chipuk et al., 2010). To follow on, *Bcl-2* was detected predominantly in differentiated tumors (Sadjadi et al., 2009). The recent availability of reagents able to detect the expression of *Bcl-2* and related genes has substantially contributed to the understanding of some of the genetically controlled mechanisms that regulate active cell death and to the investigation of the role of pro- and anti-apoptotic proteins in determining the cellular response to cytotoxic drugs, hormonal agents and radiation (Yan et al., 2009; Moul et al., 2012). The main goal of this study was to explore the *Bcl-2* expression pattern in tumor samples from the respective gastric tissues and compare the results to marginal samples as a control.

Materials and Methods

Sample collection and processing

Ten patients with gastric cancer and ten normal control individuals from Sari' hospital, Mazandaran, with certificate of ethic committee of Mazandarn University of Medical Sciences, were considered for this study from 2012 to 2013 and the samples obtained by means of endoscopy at Tooba expert clinic of Mazandaran university of medical sciences, Sari, Iran.

Two tissue samples were taken from each patient during endoscopy. One sample from tumoural section

and one sample from non-tumoural(marginal) section of stomach tissue of the same patient. As negative control, normal samples were taken from peoples whose stomach tissues were reported non-tumoral after endoscopy. Hence the expression of *BCL2* compared relatively between three groups of samples tumoral, nontumoral(marginal) and negative control.

Our experiments was performed by Qiagen RNA later, RNeasy plus mini kit from Qiagen(Qiagen, Hilden, Germany). and Revert Aid First Strand cDNA Synthesis Kit (Fermentas,Germany). The primers and probes were synthesized by Bioneer((Bioneer, South Korea) and Quanti Fast SYBR Green PCR Master Mix obtained from Qiagen (Hilden, Germany).

Total RNA isolation

The biopsy specimens were immediately stored in RNA later solution and stored at -20°C until isolation of RNA process. Total RNA isolation was performed using the method of RNeasy® Plus Mini Handbook(Qiagen,2010). For this purpose, approximately 30 mg RNA later stabilized tissue was disrupted by using a mortar and pestle followed by homogenization using needle and syringe and then the weighed (RNA later stabilized) tissue immediately placed in liquid nitrogen and grinded thoroughly with a mortar and pestle(Mowla et al., 2005). Further processing was performed following the manufacturer's protocol of RNeasy Plus Mini Kit(Qiagen, Hilden, Germany). Also a DNase treatment has been included in procedure for elimination of any genomic DNA (Qiagen, Hilden, Germany). Extracted RNAs were eluted in 40 µl RNase-free water and stored at -80°C. The quality of RNA was verified by agarose gel electrophoresis and the concentration of each RNA sample was measured at A260 using the Pico Drop 2000 (Thermo Fischer Scientific Inc) (Mowla et al., 2005).

cDNA synthesis

For reverse transcription of total RNA to complementary DNA (cDNA). the cDNA synthesis kit(Fermentas Germany,) has been used. Evaluation of concentration and purity of cDNA was performed using Pico Drop 2000 (Thermo Fischer Scientific Inc) (Behjati et al., 2005; Mowla et al., 2005; Mistry et al., 2008).

Conversion of RNA to cDNA has been done by mixing and briefly centrifuging all components after thawing, kept on ice: *i*) the following reagents were added into a sterile, nuclease-free tube on ice as following order: Then the 4 µl of Template RNA was added mixed gently, centrifuged briefly and incubated at 65°C for 5 min.(use Thermocycler Machine) chilled on ice. *ii*) Secondly, the composition was created.

After mixing the components by the pipette,they were briefly centrifuged.Then the first composition was added to this mixture.in last stage, and 1 µl revertaid M-MuLV

Table 1. Sequences of the PCR Primer Sets

Amplicon Size (bp)	Reverse Primer	Forward Primer	GenBank accession no.	Gene
126	GCCGATCCACACGGAGTACT	GGCACCCAGCACAAATGAAG	NM_10277	β-Actin
240	TGAGCAGAGTCTTCAGAGACAGCC	ATGTGTGTGGAGAGCGTCAACC	NM_000633	BCL2

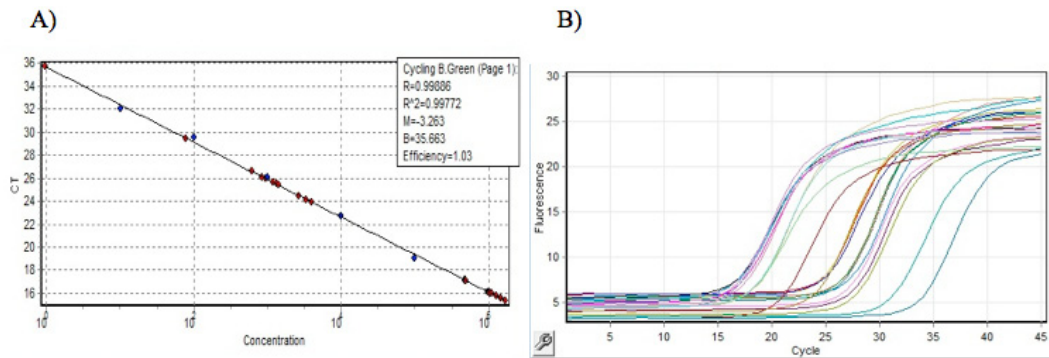


Figure 1. A) Standard Curve; B) Amplification Curves of cDNA Tissue Samples of Gastric Cancer of *bcl2* and β -Actin

reverse transcriptase enzyme was added and then centrifuged. *iii*) The mixture incubated for 5 min at 25°C followed by 60 min at 42°C. *iv*) The reaction terminated by heating at 70°C for 5 min.

Polymerase chain reaction in real time (Real-Time PCR)

Quantitative real-time RT-PCR was performed using specific primers for *BCL2* and Housekeeping β -Actin gene (chosen as a reference) (Table 1) with the Quanti Fast SYBR Green PCR Master Mix (Qiagen, Hilden, Germany) (Karasek et al., 2002) and run on the Rotor-gene 6000 (Qiagen, Hilden, Germany). Primers were designed with Beacon Designer 7 software based on the full human *BCL2* cDNA sequences (Gen-Bank™ Accession No. NM_000633) and β -Actin as housekeeping gene (Gen-Bank™ Accession No. NM_10277)(table1). The suitable annealing temperature for primers determined with multiple set up reactions in gradient phase 60°C.

Quantitative real-time PCR reactions were performed in a 10 μ L volume containing 5 μ L of QuantiTect SYBR Green PCR master mix 1X (Qiagen, Hilden, Germany), 1 μ L of forward and reverse primers and 1 μ L of first strand cDNA according to manufacturer's procedure (Karasek et al., 2002). After an initial 5-min heating at 95°C as activation step, 40 cycles of denaturation at 95°C, 10 Sec; annealing and extension at 60°C, 30 Sec have been carried out. In each PCR run, preparation of standard curve was carried out by serial dilution of cDNA from each sample. *BCL2* gene mRNA expression was assayed by comparison of the test with standard curve of the specific target and housekeeping gene in each PCR run. Also Melting curve has been designed to show confidence of production.

Statistical analysis

All measurements were done at least duplicates and Data were analyzed using GLM procedures of SAS software version 9.1. Differences between means were tested using Tukey test. Differences were considered to be significant at $P < 0.05$.

Results

Amplification stage

The standard curve which plotted based on the logarithm of the concentration of cDNA and threshold cycle (Ct) was linear (Figure 1A). Also the curves of

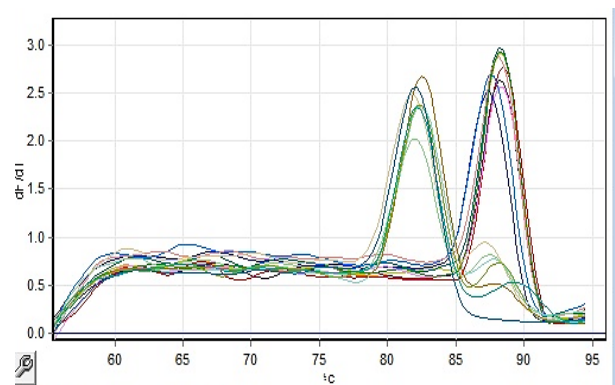


Figure 2. Melting Curve without Primer Dimers Showing Specific Amplification of *BCL2* and β -Actin Genes on Real-time RT-PCR

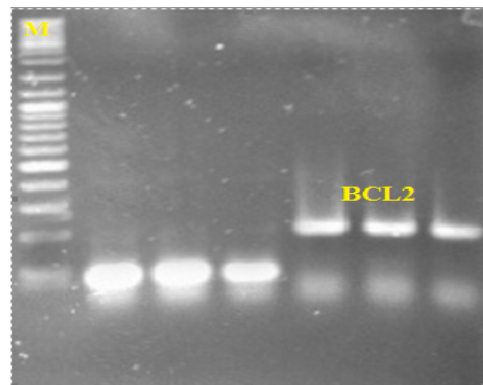


Figure 3. Products of Real-time PCR on Agarose gel (2%) Contains β -Actin and *BCL2* Genes Polymerization Product

amplification of *BCL2* and β -Actin gene using Real-time PCR showed that the amplification has performed suitably with appropriate performance (Figure 1B). Results of melting curve for the designed primers showed that each pair of primers acted as especial shape and didn't have any non-especial patches and secondary structures (Figure 2). With running Real-time PCR product on agarose gel, good qualitative results has been obtained and showed high amplification of the *BCL2* gene (Figure 3).

BCL2 expression results.

Expression results showed that *BCL2* gene was expressed in each of three types of tissues (control, tumoral and nontumoral (marginal)). The level of the expression

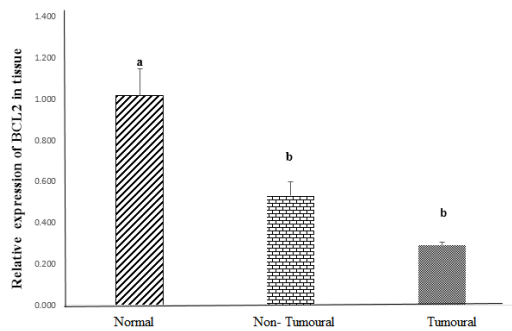


Figure 4. Relative Expression of BCL2 in Tissue Samples of Non Tumoural(marginal), Tumoural and Normal

of *BCL2* gene in control group increased compared with non tumoural marginal and tumoural group. Although the expression of *BCL2* gene in control groups were increased compared with both tumoural and non-tumoural(marginal) groups ($P>0.05$) [Figure 4].

Discussion

Identification of molecular markers that provide an insight into the potential behavior or aggressiveness of tumors is a necessary step for the improvement of cancer treatment. *Bcl-2* protein product seems to be one of the most promising members of molecular markers to evaluate cancer malignant behavior. The *Bcl-2* gene is a protooncogene whose protein product inhibits apoptosis. Its role is associated with keeping cells alive, but not by stimulating them to proliferation, as other protooncogenes do (Sulkowska et al., 2003). This study has been aimed to investigate the expression of *BCL2* gene in people stricken by gastric adenocarcinoma and to compare this expression between patient and normal individuals. High *BCL2* gene expression was recorded in normal samples while cancer samples showed significant low rate of *Bcl-2* gene expression. *BCL2* proteine through interactions with BAX proteine to inhibit apoptosis and also cell cycle arrest. So *BCL2* has a dual role. On one hand *BCL2* plays an important role as an oncogene to inhibit apoptosis; on the other hand, it can initiate cell cycle arrest at G0 stage (Zinkel et al., 2006). the association between its expression and patient survival result is quite conflicting and seems tissue-specific. For example: The different results in CLL and breast cancer may be determined by the balance between the dual function of *Bcl-2* protein (Callagy et al., 2008). These findings were in parallel with those results in colorectal cancer, breast cancer, non-small-cell lung cancer, renal cancer and head and neck cancer. (Ofner et al., 1995; Silvestrini et al., 1996; Zhang et al., 1998; Fontanini et al., 1995; Hirata et al., 2009; Lehnerdt et al., 2009). Accordingly in those studies Increased *Bcl-2* expression is associated to favorable outcome. While its high expression is associated with poor survival in B-cell chronic lymphocytic leukemia (CLL), prostate cancer and urinary tract transitional cell cancer(Faderl et al., 2002; Keshgegian et al., 1998; Stackhouse et al., 1999). Rostamizadeh et al demonstrated the higher expression levels of the *Bcl-2* gene in tumor samples compared with

marginal samples, however, this was not a significant difference ($p>0.05$). (Rostamizadeh et al., 2013). But, In our study The level of the expression of *BCL2* gene in control group increased compared with non tumoural marginal and tumoural group. According to our results there was significant difference between the levels of *BCL2* expressions in patients compared with healthy individuals .

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