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Detection of Bcl-2 Gene in Leukaemic Rats Using an EvaGreen Real-time RT-PCR Assay

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ABSTRACT

Bcl-2 is an anti-apoptotic gene that is involved in the apoptosis process. Suppression of apoptosis by anti-apoptotic gene can contribute to the occurrence of diseases such as leukaemia. The objectives of this study were 2-folds: first, to compare the sensitivity of an EvaGreen quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) with a conventional RT-PCR for the amplification of the Bcl-2 gene; second, to determine the expression of the Bcl-2 gene in *N*-methyl-*N*-nitrosourea (MNU)-induced leukaemia in rats using the EvaGreen qRT-PCR assay. A total of 32 male Sprague Dawley rats were assigned into two groups (n=16), namely, control and MNU groups. In particular, MNU was administered intraperitoneally (i.p) at a dose of 60 mg/kg body weight per injection at two times per week for 2 consecutive weeks. The rats were sacrificed after five months and blood samples were collected for RNA extraction and haemogram. The RNAs were converted into cDNA and amplified using both the EvaGreen qPCR and the conventional PCR assays. All the results were normalised with a housekeeper gene, i.e. glyceraldehyde 3-phosphate dehydrogenase (GADPH). The products of amplification were run on gel electrophoresis and all the results were then compared. Based on the relative intensity of the bands, the EvaGreen qRT-PCR assay was highly sensitive compared to the conventional RT-PCR assay as the Bcl-2 gene could not be amplified using the conventional RT-PCR. Interestingly, the results in this study showed that the expression of Bcl-2 was higher in rats with marked lymphocytosis as compared to the leukaemic rats with normal to mildly increase in lymphocyte count. In conclusion, EvaGreen qRT-PCR assay is more sensitive compared to the conventional RT-PCR, and Bcl-2 gene is abundantly expressed in leukaemic rats with marked lymphocytosis compared to the leukaemic rats with normal to mildly increase in lymphocyte number.

Keywords: Bcl-2, leukaemia, *N*-methyl-*N*-nitrosourea (MNU), EvaGreen qRT-PCR, conventional RT-PCR assays

INTRODUCTION

Apoptosis or programmed cell death is defined as a mechanism of cellular suicide which occurs after sufficient cellular damage. Apoptosis differs from necrosis and it is involved in the development of many diseases, such as cancer (Arends & Wyllie, 1991; Nikitakis *et al*., 2004; Loro *et al*., 2003; 2005). Various imbalances in the apoptotic system, such as insufficient

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amount of apoptosis, can lead to the development of autoimmunity and lymphoma by failing the process of lymphocyte death (Maniati *et al*., 2008). Apoptosis genes are thought to be useful as cancer biomarkers as they mark the expression of genes that control the apoptosis process. A group of proteins called B-cell lymphoma 2 (Bcl-2) family control the apoptosis process in cells (Goodsell, 2002). In general, Bcl-2 family proteins can be divided into proapoptotic and anti-apoptotic genes. Meanwhile, Bcl-2 functions as anti-apoptotic gene as it exhibits the ability to inhibit the apoptosis process and known as the most important gene of the Bcl-2 family (Reed, 1994; Lu *et al*., 1996). The Bcl-2 gene encodes a membrane protein localised in the nuclear membrane, the inner surface of mitochondria, and the endoplasmic reticulum (Armstrong, 2006), and it was first discovered by analysis of the t(14; 18) chromosomal translocation associated with human follicular B-cell lymphoma (Tsujimoto *et al*., 1985; Cleary *et al*., 1986).

In this study, the induction of leukaemia in male Sprague Dawley rats was performed by injection of *N*-methyl-*N*-nitrosourea (MNU). *N*-methyl-*N*-nitrosourea (MNU) was found to have induced tumours in many parts of the studied animals (Koestner *et al*., 1977; Kunze *et al*., 1989; Maekawa *et al*., 1985; Ogiu *et al*., 1977; Tsuda *et al*., 1983; Bosland & Prinsen, 1990; Hazilawati *et al*., 2010; Hutheyfa *et al*., 2011), as it is a very powerful carcinogen. In addition, it is also an alkylating agent, and exhibits its toxicity by transferring its methyl group to nucleobases in nucleic acids. Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) was chosen as a method of analysis for the transcription of Bcl-2 in this study. This technique has become an established procedure for quantifying the levels of gene expression, as well as gene rearrangements, amplifications, deletions or point mutations (Ponchel, 2007). Some examples of the chemistries available for qPCR are SYBR Green I and EvaGreen dyes. They are fluorogenic dyes that exhibit little fluorescence when in solution, but emit a strong fluorescent signal upon binding to double-stranded DNA.

The aims of the present study were to compare the sensitivity of the EvaGreen qRT-PCR assay with the conventional RT-PCR, and to determine the expression of Bcl-2 gene in the MNU-induced leukaemic rats using the method developed above.

MATERIALS AND METHODS

Animals and Experimental Design

A total of 32, 6-week-old, male Sprague Dawley rats were purchased from the Laboratory Animal Resource Unit, Faculty of Medicine, Universiti Kebangsaan Malaysia (UKM) in Bangi, Selangor and placed in an individual polycarbonate rat cage in a colony animal room maintained at 22-27°C, with 40-70% humidity, and 12-hour light/12-hour dark conditions. The animals were acclimatised for two weeks and given commercial rat pellets and water *ad libitum* prior to the experiment. The rats were divided into 2 groups (n=16), namely control and MNU group. The rats in the control group were injected with normal saline intraperitoneally (i.p), whereas those in the MNU group were injected with *N*-methyl-*N*-nitrosourea (MNU) (Sigma-Aldrich, USA) dissolved freshly in normal saline, i.p, twice a week for 2 consecutive weeks at a dose of 60 mg/kg/body weight per injection (with a total dose of 240 mg/kg body weight). The animals were humanely sacrificed by bleeding under anaesthesia after five months. Their blood samples were collected from the heart via cardiac puncture for RNA extraction. These blood samples were also collected and examined for the evidence of leukaemia by Hutheyfa *et al*. (2009).

RNA Extraction

Total cellular RNAs were extracted using a QIAamp RNA Blood Mini Kit (Qiagen, USA) according to the manufacturer's instruction, from fresh blood collected in heparinised tubes. The optical density (OD) of the extracted RNA

was determined by measuring the absorbance at A260/A280 nm using a spectrophotometer (Eppendorf, German) and kept in -80°C until reverse transcription for cDNA synthesis.

Amplification of Bcl-2 gene Using a qRT-PCR Assay

The extracted RNA was converted into cDNA using a GeneAmp RNA PCR Core Kit (Applied Biosystems, USA) as per manufacturer's instruction. Master mix for qPCR was prepared with total reaction mixture of 20 uL contained 10 uL ImmoMix™ (Bioline, USA), 1 uL EvaGreen™ Dye (Biotium, USA), 1.5 um Bcl-2 forward and reverse primers (1st BASE, Malaysia), and 2uL of the sample containing 3 - 4 ng of cDNA mixed with sterile deionised water in a single real-time PCR tube for each reaction. The conditions for Bcl-2 amplifications were as follows: 1 cycle of 95°C for 10 minutes (predenaturation), followed by 37 cycles of 94°C for 20 seconds (denaturation), 64°C for 30 seconds (annealing), and 72°C for 45 seconds (extension). The amplification was done using a real-time PCR machine (Bio-Rad CFX96).

The following Bcl-2 primer sequences specific for rats, which were previously described by Brambrink *et al*. (2000), were used: Bcl-2 antisense, 5'-TTTCATATTT GTTTGGGGCAGGTC-3' and Bcl-2 sense, 5'-ATGGGGTGAACTGGGGGAGGATTG-3'. The size of PCR product is 350 bp.

Amplification of Bcl-2 and GAPDH Genes Using Conventional RT-PCR Assay

The same cDNA samples converted from the extracted RNA were used. Master mix for the conventional PCR was prepared using sterile deionised water with total reaction mixture consisted of 20 uL containing 2 mM $MgCl_2$ solution, 1× PCR Buffer II, 200 uM for each dNTPs (Applied Biosystems, USA), 1.5 uM Bcl-2 or GAPDH forward and reverse primers (1st BASE, Malaysia), 0.05 U AmpliTaq DNA polymerase (Applied Biosystem, USA) and 2 uL of sample containing 3 - 4 ng of cDNA. The amplification conditions for Bcl-2 gene were as follows: 1 cycle of 95°C for 2 minutes (predenaturation), followed by 37 cycles of 95°C for 30 seconds (denaturation), 61.4°C for 30 seconds (annealing), 72°C for 30 seconds (extension), and 1 cycle at 72°C for 7 minutes for the final extension. Similar amplification conditions were used for the GAPDH gene with 29 cycles and an annealing temperature of 55°C. The amplification of the cDNA was performed using a conventional PCR machine (Little Genius, BIOER).

Similar Bcl-2 primers used in the qRT-PCR were used for the conventional RT-PCR. The following primer sequences were used for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene; GAPDH sense: 5'-TGTGAACGGATTTGGCCGTA-3' and GAPDH antisense: 5'-CATTTGA TGTTAGCGGGATC-3' (GenBank accession number of the sequence is NM 017008) with the PCR product of 240 bp (designed using the Oligo Primer Analysis Software v.7).

Gel Electrophoresis

Ten microlitre of Bcl-2 qRT-PCR, Bcl-2 conventional RT-PCR and GAPDH conventional RT-PCR products were electrophoresed at 80V for 60 minutes in 1.5% agarose gel, stained with 0.5 ug/mL ethidium bromide solutions for 15-20 minutes and visualised under UV transillumination (Alpha Imager, GEL DOC).

RESULTS AND DISCUSSION

Several studies have shown that qRT-PCR is highly sensitive compared to conventional RT-PCR (Di Trani *et al*., 2006; Gurukumar *et al*., 2009). In spite of its sensitivity, the results produced by qRT-PCR are more rapid as gel electrophoresis is no longer necessary. However, qRT-PCR can be highly expensive, depending on the types of dye used. Hence, attempt was made to develop and optimise conventional RT-PCR, which is less expensive. The results of this study showed Bcl-2 was unable to amplify using the conventional RT-PCR assay for both the control

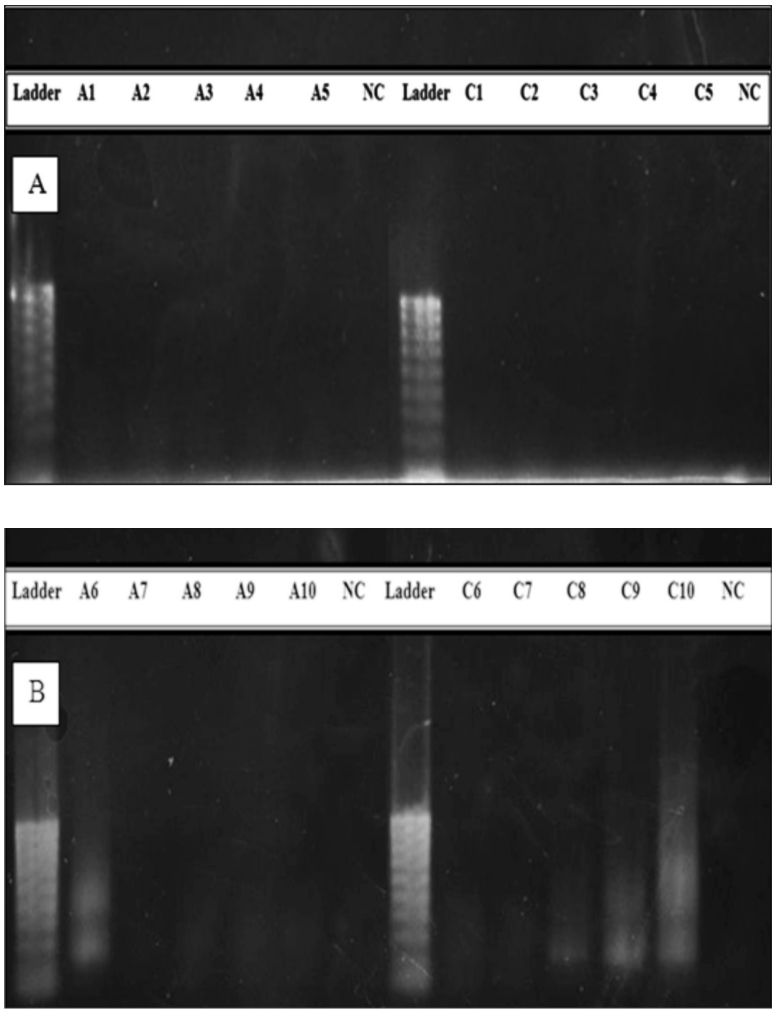

Fig. 1A and B: Bcl-2 gene amplification products of control (A1, A2, A3, A4, A5, A6, A7, A8, A9, and A10) and leukaemic (C1, C2, C3, C4, C5, C6, C7, C8, C9, and C10) rats amplified using conventional RT-PCR. 100 bp DNA markers (Bioline, UK) are shown in lanes 1 and 8 for each picture. Negative controls (NC) show no amplification. Note that no bands were amplified in A, whereas a few unspecific PCR products were amplified in B

and leukaemic rats (*Fig. 1A* and *1B*). The results were normalised by further analysis of the cDNA of the control and leukaemic rats using GAPDH primer via conventional RT-PCR, whereby the total RNA was clearly shown as succesfully extracted (*Fig. 2A* and *2B*).

Meanwhile, haematological results revealed that all rats induced with MNU had leukaemia with 30% and 70% of the rats had marked lymphocytosis and normal to mildly increase in lymphocyte count, respectively (Hutheyfa *et al*., 2009). The expression of Bcl-2 gene of the leukaemic rats was determined via gel electrophoresis of the EvaGreen qRT-PCR products (*Fig. 3*). The finding showed that leukaemic rats with marked lymphocytosis (C1 to C5) had brighter gel bands intensity compared to the leukaemic rats with normal lymphocyte

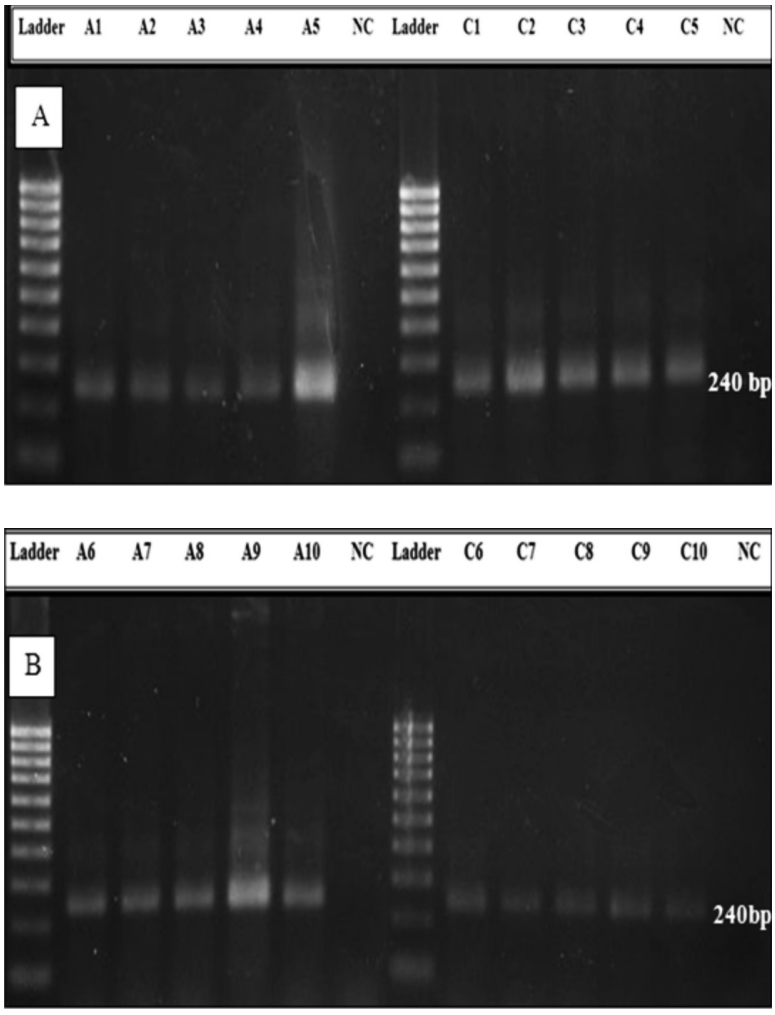

Fig. 2A and B: GAPDH gene amplification products (240 bp) of the control (A1, A2, A3, A4, A5, A6, A7, A8, A9, and A10) and leukaemic (C1, C2, C3, C4, C5, C6, C7, C8, C9, and C10) rats amplified using conventional RT-PCR. 100 bp DNA markers (Bioline, UK) are shown in lanes 1 and 8 for each picture. Negative controls (NC) show no amplification. Note that all RNAs extracted from the blood samples of the control and leukaemic rats had been successfully extracted

count (C6 to C10). This indicates that the expression of Bcl-2 gene was significantly higher in leukaemic rats with lymphocytosis. The results are consistent to the previous reports which demonstrated higher expression of Bcl-2 in patients diagnosed with high-grade breast cancer (Veronese *et al*., 1998), Burkitt lymphoma (Carbone *et al*., 2010) and advanced stage of colon cancer (Bousserouel *et al*., 2010).

In conclusion, Bcl-2 gene is highly upregulated in leukaemic rats with lymphocytosis, while EvaGreen qRT-PCR assay is highly sensitivity compared to conventional RT-PCR for the amplification of the Bcl-2 gene in leukaemic rats.

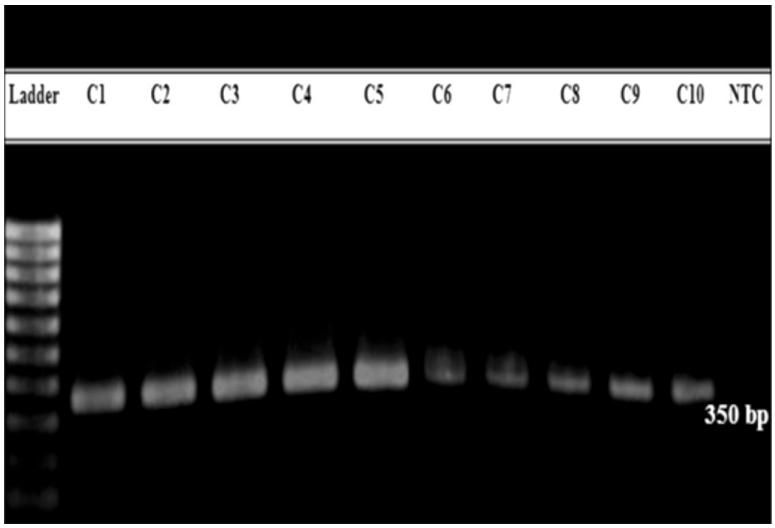

Fig. 3: Bcl-2 gene amplification products (350 bp) of the leukaemic rats amplified using EvaGreen qRT-PCR assay. 100 bp DNA marker (Bioline, UK) is shown in the first lane. Lanes C1 to C5 are the samples from rats with acute leukaemia, whereas lanes C6 to C10 are the samples from the rats with chronic leukaemia. The last lane is no template control (NTC). Note that brighter bands are observed in lanes C1 to C5 and faded bands are observed in lanes C6 to C10

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