Expression of Bax and Bcl2 Genes in Peripheral Blood Lymphocytes of Patients with Differentiated Thyroid Cancer

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Abstract

Context: Thyroid cancer is the most common endocrine malignancy worldwide. Iodine-131 is used in the treatment of thyroid cancer with dosage of 100 mCi. In the medical applications of ionizing radiation besides the advantages such as diagnosis and treatment of diseases, the risks arising from exposure should be considered as well. Aims: The present study aimed to evaluate the changes in expression levels of apoptotic Bax and Bcl-2 and the ratio of Bax/Bcl-2, in the peripheral blood lymphocytes (PBLs) of patients with differentiated thyroid cancer (DTC). Settings and Design: This study was conducted on fifty thyroid cancer patients who had undergone surgery and were under treatment with 100 and 150 mCi doses. Subjects and Methods: Blood samples were taken from the patients, one before iodine treatment and another 48 h after therapy. Bax and Bcl-2 gene expression levels were measured by using real-time reverse transcriptase polymerase chain reaction. Statistical Analysis Used: The data were analyzed using one-way analysis of variance followed by samples t-test and independent samples t-test. Results: Significant changes were observed in the percentage of apoptotic cells, in groups, after radioiodine therapy compared with before treatment. The ratio of Bax/Bcl-2 in both groups showed a significant increase ($P < 0.001$). The relative expression level of Bax gene showed a significant increase in comparison with the control group. Conclusions: Iodine therapy reduced expression of Bcl-2 and a significant expression of Bax and finally increased the ratio of Bax/Bcl-2. Iodine therapy led to apoptosis in the PBLs of patients with DTC. Therefore, it can be suggested that this method can be useful for monitoring and detecting destructive effects of ionizing radiation in nuclear medicine patients.

Keywords: Bax, Bcl2, differentiated thyroid cancer, gene expression, peripheral blood lymphocytes

INTRODUCTION

The differentiated thyroid cancer (DTC) comes for 1%–2% of malignant cancer in human. Its annual incidence is estimated 2–3.8 for women and 1.2–2.6 for men/100,000 persons. Its outbreak has increased in the recent decades.[1]

This cancer derives from either the classified epithelial follicular cells in the form of cancer with high differentiation (including papillary cancers and thyroid follicular, the thyroid cancer with low differentiation and anaplastic) or the parafollicle cells as generator of calcitonin (such as medullary cancer).[2] Generally, the differentiated forms of thyroid cancer have a slow progression trend; their treatment includes taking measures such as surgery, using radioactive iodine, and inhibiting TSH hormone.[2] Radioactive iodine-131 has been used in the treatment of malignant thyroid since 1940.[3] The radioactive 131 iodine is one of the iodine isotopes with half-life of 8 days and is released by the distribution of beta particles along with gamma radiation; and changes into stable xenon 131.[4] In the medical applications of ionizing radiation besides the advantages such as diagnosis and treatment of diseases, the risks arising from exposure should be considered too.[5] Ionizing radiation has been known as causing oxidative stress by generating active oxygen types and free radicals in the tissues and irradiated cells.[6] These free radicals react with DNA, existing lipids in the nucleus and membrane.[6,7] Significant changes in the structure and practice of DNA (failure of double-stranded DNA) and membrane (hard)
lead to cell death through apoptosis.\textsuperscript{8,9} Apoptosis is induced by a variety of toxic insults including growth factor deprivation and ionizing radiation.\textsuperscript{10} Ionizing radiation damages DNA and is one agent that induces apoptosis in certain cells, including human lymphocytes. In the early stages of apoptosis, it is measured.\textsuperscript{11,12} Phosphatidylinositol serine transfers from the inner surface to the outer surface of the cell membrane and the integration of cell membrane has been preserved. In the late apoptosis stages of apoptosis, the integration of cell membrane is destroyed, and the time of cell death or apoptosis depending on the type of cell has been estimated from a few hours to a few days.\textsuperscript{13,14} The programmed cell death precisely depends on action and reaction of some gene products that activate or inhibit the process of cell suicide. Two large gene families including Caspases and Bcl-2 are involved in the path of apoptosis.\textsuperscript{15} The Bcl-2 family has two protein groups including apoptosis promoter and inhibitor of apoptosis. The average ratio of these proteins determines a cell destiny. The Bcl-2 protein is placed in the cover of core and mitochondria; and it functions through the connection to Bax.\textsuperscript{16} The Bax protein in terms of structural is Bcl-2 homologous, and it is considered to be a promoter of apoptosis that is placed in the cytoplasm or the cellular membrane, and hence, in terms of performance, this antagonist protein has a protective role for Bcl-2. The caspase molecules are generated in the form of prefabricate and just after receiving a death signal by cell, they are activated.\textsuperscript{17}

Caspase 3 is an executive caspase that is activated by the starter caspases and breaks the survival and integrity of proteins. The death signals cause the setting up the apoptosis proteins family of Bcl-2, especially Bax. These proteins cause the release of cytochrome C from mitochondria to out. The release of mitochondrial cytochrome C into the cytoplasm and its subsequent association with the Apaf-1 protein is thought to be an absolute requirement for the activation of caspase-9, the apical caspase in the mitochondrial pathway of apoptosis.\textsuperscript{18,19}

In another study in 2013, conducted by Verne Dick et al., apoptosis and micronuclei (MN) were investigated as the reflection of cytogenetic injury in the blood lymphocyte of 18 patients suffered from thyroid cancer under treatment with 100 and 150 mill curie doses. As apoptotic cells, using Annexin V-FITC/7-AAD kit and frequency of MN was assessed by MN assay. In this study, after radiiodine therapy in thyroid cancer patients, increase of primary apoptosis and MNs was observed.\textsuperscript{20}

The aim of this study was to investigate the expression pattern of some inhibitors of Bcl-2 cell death and inducers of Bax cell death in patients under iodine therapy with different doses.

**Subjects and Methods**

This research was approved by the ethical committee of Shiraz University of Medical Sciences. The consent form was presented to patients and voluntarily read and signed. The studied population included fifty patients with thyroid cancer with the mean age of 39.08 ± 9.5; they had undergone thyroidectomy and were under treatment in the nuclear medicine department of Shiraz Namazi Hospital. Blood samples were taken before radioiodine therapy and 48 h after iodine therapy. Blood samples (2 ml of peripheral blood from patients) were collected in heparinized tubes. These samples were used for the measurement of Bax and Bcl-2 expression levels using real-time (RT) reverse transcriptase polymerase chain reaction (PCR). At first, separation of lymphocytes was made using the ficoll of innotraining, Germany, based on the standard protocol. The blood was washed with the buffer of phosphate saline with the same volume and then, 4 ml volume (blood and phosphate-buffered saline [PBS]) was added to 2 ml ficoll and centrifuged for 20 min with 3000 RPM. The separated lymphocytes after three times of washing with PBS were centrifuged for 10 min in 1400 RPM g. The surface layer was thrown out and the extracted lymphocytes were mixed with 500 PBS µl.

**RNA preparation and quantification**

After isolation of the lymphocytes from the peripheral blood samples, total RNA was extracted from blood samples with the RNX plus extraction kit (CinnaGene, Iran), according to the manufacturer’s protocol and stored at −20°C. For extracting of RNA from chloroform and isopropanol and wash it, 75% ethanol was used. The concentration and quality of RNA were determined by measuring the absorbance by spectrophotometer system (Bekman, USA) at 260 nm (A260) and A260/A280 ratio, respectively.

**cDNA synthesis and real-time quantitative polymerase chain reaction**

Before cDNA synthesis, to remove pollution of RNA with DNA, the DNase I kit (Thermo Science, USA) was used. The synthesis of cDNA was done by using RevertAid First Strand cDNA Synthesis kit (Fermentase, lithuania) and based on the manufacturer’s instruction. The used designed primers of Bax, Bcl-2, and B2 m gene are shown in Table 1. The B2 m gene was used as the endogenous reference. The

**Table 1: Sequence-specific primers for genes BCL-2 and BAX and B2m**

| Gene name | Sequence of forward primers | Sequence of reverse primers | Product size (bp) |
|-----------|-----------------------------|-----------------------------|-------------------|
| BAX       | CTTCACGGTTTCTCCTACCCAG       | CTCCATGTACTGTTCCAG          | 169               |
| BCL-2     | ATGGATGGGAATGTGTGCCTTATGCA  | CCCCAGCATGACAGCAGGAAA       | 153               |
| B2m       | GTATGCCTTCGCGTGAAC          | AACCTCCATGATGCTGTTAC        | 87                |
RT quantitative-PCR (QPCR) was done by CYBR Green kit (yekta tajhiz, Iran). The QPCR were done in cycle including: 2 min at 95°C for initial denaturation, and then, forty cycles of denaturation for 30 s in 95°C, annealing for 40 s in 57°C, with extension for 30 s in 72°C. The PCR products were separated on 2% agarose gel, and they were visible with ethidium bromide; then, they were evaluated by using trans-luminator advice UV (Uvidoc, UK). Target genes were quantified relative to the reference gene using the mathematical model described by computer tomography.

**Verification polymerase chain reaction and primer design**

To verify Bax, Bcl-2, and B2 m primers with 169, 153, and 87 base pair (bp) band lengths, PCR was performed with a cDNA sample. Five microliter of PCR product was loaded on 2% agarose gel. The distinctly mentioned band lengths of each gene were visible after ethidium bromide staining. Figure 1 depicts the electrophoresis image of the three primers Bcl-2 -Bax and B2 m (Bcl-2, 153 bp, Bax, 153 bp, and B2 m 87 bp).

**Statistical methods**

The data were analyzed using one-way analysis of variance followed by samples t-test and independent samples t-test. The values of $P < 0.05$ were considered statistically significant.

**RESULTS**

The changes in relative expression level of Bax and Bcl-2 genes Figure 2 shows the relative expression level of Bax gene 48 h after iodine therapy, at a dose of 100 and 150 mci. The relative expression level of Bax gene at a dose of 100 and 150 mci showed a significant increase in comparison with before iodine therapy ($P = 0.0001$).

As observed in Figure 3, in two groups a significant decrease was found in the expression level of Bcl-2 in comparison with the control group ($P < 0.05$). The relative expression level of Bcl2 gene at a dose of 100 and 150 mCi showed a significant reduction in comparison with before iodine therapy.

Figure 4 shows the relative expression level of Bax/Bcl-2 ratio 48 h after the iodine therapy at doses of 100 and 150 mCi). The ratio of Bax/Bcl-2 in after iodine therapy in comparison with before treatment led to a significant increase in the Bax/Bcl-2 ratio.

Figure 5 shows the relative expression level Bax, Bcl2, and Bax/Bcl-2 ratio 48 h after iodine therapy at different doses. At a dose of 150 mCi after iodine therapy, a significant increase was showed in the expression level of Bax comparison with dose 100 mci. We observed a significant increase in the ratio of Bax/Bcl-2 at a dose of 150–100 mci. It can be concluded that, at higher doses, expression of the apoptotic gene will increase and the percentage of apoptotic cells will be greater. Tables 2 and 3 show the relative expression of Bax and Bcl-2 genes and Bax/Bcl-2 ratio of two therapeutic doses 48 h after iodine therapy. The relative expression level of Bax gene in both groups showed a significant increase after radioiodine therapy compared with before treatment. Moreover, the relative decrease in gene expression of Bcl-2, after the iodine treatment in both groups was statistically significant ($P < 0.05$). The ratio of the Bax/Bcl-2 in both groups showed a significant increase ($P < 0.05$).

As shown in Table 4, we examined the effect of two different doses of the gene expression of apoptosis. At a dose of 150 mCi, there was an increase in the dose of 100 mCi of the Bax gene expression. Bcl-2 levels also significantly decreased.
undergone thyroidectomy is applied for the destruction of the remaining thyroid with probable metastases. Lymphocytes are easily eliminated as high-sensitivity cells in patients treated with radioactive iodine. Apoptosis is a reaction of cytogenic damage in the cells, and in this study, the expression of apoptosis genes was compared before and after iodine therapy. The purpose of this study was to investigate the effect of iodine therapy on the changes of apoptosis levels and expression of apoptosis genes.

Based on the obtained results of this study, the percent of apoptosis lymphocytes, 48 h after iodine therapy in comparison with before treatment shows that the increased level can be attributed to the increase in Bax gene expression in both groups which received 100 and 150 mCi. In addition, increase in the expression of the ratio of Bax/Bcl2 is an effective factor for incidence of apoptosis.

According to the results of a study conducted by Cui et al., it was shown that after 4 h exposure of all the body to 2–8 gray gamma radiation, the apoptosis lymphocytes increased rapidly, and expression of Bax gene in 24 h after radiation reached its maximum amount. The Bcl-2 expression reduces after passing 3 h after radiation, and the reduction reaches its minimum amount 24 h after that. This finding is consistent with the obtained result of our research. MN and apoptosis are raised as cytogenic damages arising from radiation. The study results showed iodine therapy caused the effect of apoptosis, and its gene expression.

**Table 2: Comparison of gene expression BCL-2 and BAX before and 48 hours after radioiodine therapy in the dose of 100 mCi**

|          | Before Iodine-131 | After Iodine-131 | Significance (P) |
|----------|-------------------|------------------|------------------|
| BAX      | 1.07±0.24         | 1.89±0.59        | 0.0001           |
| BCL-2    | 1.02±0.29         | 0.78±0.22        | 0.03             |
| BAX/BCL-2| 1.16±0.50         | 2.69±1.33        | 0.0006           |

**Table 3: Comparison of gene expression BCL-2 and BAX before and 48 hours after radioiodine therapy in the dose of 150 mCi**

|          | Before Iodine-131 | After Iodine-131 | Significance (P) |
|----------|-------------------|------------------|------------------|
| BAX      | 1.11±0.29         | 2.01±0.68        | 0.0001           |
| BCL-2    | 1.03±0.32         | 0.58±0.20        | 0.0001           |
| BAX/BCL-2| 1.18±0.48         | 4.04±2.18        | 0.0001           |

**Table 4: Comparison of gene expression BCL-2 and BAX in human lymphocytes at a dose of 100 and 150 mCi**

|          | 100 mCi | 150 mCi | Significance (P) |
|----------|---------|---------|------------------|
| BAX      | 1.88±0.58 | 2±0.67  | 0.5              |
| BCL-2    | 0.78±0.22 | 0.57±0.19 | 0.005           |
| BAX/BCL-2| 2.695±1.33 | 4.03±2.18 | 0.03            |

Furthermore, we observed a significant increase in the ratio of Bax/Bcl-2 at a dose of 150–100 mci and it can be concluded that, at higher doses, expression of the apoptotic gene will increase and the percentage of apoptotic cells will be greater.

**Discussion**

The 131 iodine is applicable in the thyroid cancers with a dosage higher than 100 mCi. Iodine in the patients that have undergone thyroidectomy is applied for the destruction of the remaining thyroid with probable metastases. Lymphocytes are easily eliminated as high-sensitivity cells in patients treated with radioactive iodine. Apoptosis is a reaction of cytogenic damage in the cells, and in this study, the expression of apoptosis genes was compared before and after iodine therapy. The purpose of this study was to investigate the effect of iodine therapy on the changes of apoptosis levels and expression of apoptosis genes.
**Conclusions**

Iodine therapy reduces the relative expression of anti-apoptotic Bcl-2, and pro-apoptotic Bax gene expression was increased significantly. The ratio expression of Bax/Bcl-2 in doses of 100 and 150 mCi showed a significant increase. Apoptosis in PBLs after treatment with 100 or 150 mCi iodine-131 was significantly higher than before treatment. Patients with DTC have a significantly higher level of apoptosis in PBLs after iodine therapy. Based on the results of this study, radiiodine therapy in patients with thyroid cancer has led to increased apoptosis as a marker of cytogenetic damage.

Therefore, it can be suggested that this method can be useful for monitoring and detecting destructive effects of ionizing radiation in nuclear medicine patients.

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**Conflicts of interest**

There are no conflicts of interest.

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