Bax/Bcl-2 expression ratio in prediction of response to breast cancer radiotherapy

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A R T I C L E   I N F O

A B S T R A C T

Objective(s): Radiotherapy is one of the most effective modalities of cancer therapy, but clinical responses of individual patients varies considerably. To enhance treatment efficiency it is essential to implement an individual-based treatment. The aim of present study was to identify the mechanism of intrinsic apoptosis pathway on radiosensitivity and normal tissue complications caused by the radiotherapy.

Materials and Methods: Peripheral blood mononuclear cells from ten breast cancer patients were exposed to 6MV X-rays to deliver 1 and 2 Gy. Expression levels of *Bax, Bcl-2*, and *Bax/Bcl-2* ratio were examined by relative quantitative RT-PCR. All the patients received similar tangential irradiation of the whole breast and conventional fractionation. Skin dosimetry was done by GAFChromic EBT-3 film and clinical radiosensitivity was determined using the acute reactions to radiotherapy of the skin according to Radiation Therapy Oncology Group score. All statistical analyses were performed using GraphPad Prism, version 7.01.

Results: In the *in-vitro* experiment, *Bax* and *Bax/Bcl-2* ratios were significantly increased with 1 and 2 Gy doses ($P<0.001$ and $P<0.0001$, respectively). Herein, the notable result was a significant correlation between dose-response curve slope (as an *in-vitro* radiosensitivity index) and acute skin toxicity score following irradiation (as a clinical radiosensitivity index). There was no significant relationship between skin dose and reactions ($P>0.05$ for all patients).

Conclusion: Significant correlation between *Bax/Bcl-2* ratio determined before radiation therapy and clinical response in the patients, can be used as a biomarker to identify radiosensitive individuals. However, further studies are required to validate radiation-induced apoptotic biomarkers.

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Introduction

Radiation therapy (RT) has always been one of the major modalities of cancer therapy. In the recent years, many attempts have been made to improve uniformity of dose distribution to a planning target volume while delivering a minimum dose to the adjacent organs at risk. On the other hand, individual-based treatment should be considered to promote treatment efficiency of tumoral tissues and minimize normal tissue complications. The possibility on a personalized treatment based on anatomical features was established by employing three-dimensional conformal radiation therapy, computed tomography simulation, and magnetic resonance imaging. In doing so, intensity-modulated radiotherapy, CyberKnife, carbon ion, and proton beam therapy were developed. All these techniques are based on advancements in engineering sciences and manufacturing technology.

Despite these efforts, treatment outcomes based on patient anatomy vary from patient to patient.

Acute skin reaction (erythema or redness) was found to have a linearly increase with time during RT. The individual erythemal and tanning responses of human skin following radiation are largely genetically determined and the rate of the increase differed considerably from one patient to the other(1). Several reports have been published on patients sustaining serious damages to the tissue following a few dose fractions (2-4). Severity of acute skin reactions and the underlying causes were investigated in patients undergoing breast-conserving surgery and RT, showing no statistically significant relationship between severity of skin lesions and factors such as average tangential field size, chemotherapy, tamoxifen use, previous RT in the breast area, or skin type (5). As a result, individual factors such as inherent radiosensitivity (RS), which

seems to be the most reasonably reason, accounts for differences between patients responses. Inherent sensitivity of normal tissues to conventional RT (radiosensitive case) and primary or acquired resistance of tumor cells (radioresistant case) are serious challenges in the treatment of cancer (6, 7). Except for a few rare cases of recessive genetic disorder, ataxia telangiectasia (8), Fanconi anemia (9), ligase IV (10), and Nijmegen breakage syndrome (11), no specific phenotype has been observed in radiosensitive patients. Thus it seems to be useful to develop a predictive technique to identify radiosensitive patients. Some molecular biomarkers are currently evaluated in preclinical studies in order to establish predictors for treatment decisions in radiation oncology. Although appreciable efforts have been made to predict RT-induced acute normal tissue reactions, a few assays have been suggested, and so far no single assay has been approved to be clinically practicable. The standard method to determine RS of tumor cells is the clonogenic assay. However, this assay is not practically applied in clinical settings due to some reasons such as the needed time for colony formation. Intrinsic RS analysis includes: all the measurement methods based on DNA damage (12), chromosomal damage (13), DNA repair (14), apoptosis (15), gene expression modification (16), and DNA double-strand break (17, 18). A key mechanism in cancer therapy is that cancer cells die from the common pathway of apoptosis responses to DNA damage and that cells resistant to apoptosis are resistant to therapy (19). Thus, production of apoptosis is one of the most important endpoints used to estimate inherent RS.

Apoptotic signaling can be initiated in different cellular compartments, including the nucleus, mitochondria, and cell membrane and proceed by several routes. Mitochondrial pathway is the principal route that initiate apoptosis after radiation and determine the fate of the cell for death or survive. Permeabilization of the mitochondrial membrane is regulated by proteins of the B‑cell lymphoma‑2 (*Bcl‑2*) family to release cytochrome c and other apoptosis-activating factors (20-23). Pro-apoptotic members of *Bcl-2* family (*Bax*, *Bak* etc.) induce the release of cytochrome c and cause mitochondrial dysfunction. In contrast, anti-apoptotic members such as *Bcl‑2* work as protectors of the outer membrane and preserve its integrity by suppressing the release of cytochrome c (24). Thus, a critical determinant of the intrinsic apoptosis pathway is the balance between the ratio of *Bax* and *Bcl-2* genes expression that plays a role in initiation of apoptosis (25). An important regulator of

Bax and *Bcl-2* genes expression is the tumor suppressor protein *p53* that has multifunctional ability to activate cell cycle checkpoints, DNA repair mechanisms and apoptosis response for maintaining genomic stability (26). DNA damaging agents induce increased levels of *p53* that plays a leading role to directly activate pro-apoptotic *Bax* gene to engage the apoptotic program (27, 28). Given that apoptosis seems to play an important role in cell response to radiation, the current study investigated whether there is a correlation between the severity of acute skin reaction in breast cancer (BC) patients and increased apoptosis capacity. The results were expected to identify cancer patients most likely to experience severe skin reactions to RT depended on *Bax*/*Bcl-2* ratio.

Materials and Methods

Sampling

The assay was performed on blood samples collected from 10 BC patients who were involved in the study. Approval on medical ethics of the study was obtained from the Ethics Committee of Mashhad University of Medical Sciences, Mashhad, Iran.

Irradiation and clinical radiosensitivity measure

For all the patients, simulation was performed with CT simulator. The patients were treated by a 3-dimensional conformal RT technique using 6/15 MV photons generated by a linear accelerator (Siemens, Concord, CA, USA) with a dose rate of 2 Gy.Minute^{-1} for 6 MV photons and 3 Gy.Minute^{-1} for 15 MV photons. All studied patients received tangential irradiation of the whole breast with lateral and medial wedge fields and conventional fractionation (2 Gy/fraction, 5 days per week). Clinical radiation sensitivity was determined according to Radiation Therapy Oncology Group (RTOG) score for skin acute reactions (Table 1) (29).

In-vivo GAFChromic film dosimetry

In vivo dosimetry was performed by using GAFChromic EBT-3 films (International Specialty Products, Wayne, New Jersey, USA). In order to calibrate the films, twenty 2 × 3 cm^2 pieces of the film (lot # 03311403) were cut and divided into 10 groups in duplicates.

For calibration, the films were irradiated by photon beams of a 6 MV linac (Siemens, Concord, CA, USA) calibrated by an ionization chamber at a dose rate of 2 Gy.Minute^{-1}. A wide range of doses: 0.0, 0.2, 0.5,

Table 1. RTOG skin acute radiation morbidity scoring criteria

0	1	2	3	4
No change over baseline	Follicular, faint or dull erythema / epilation / dry desquamation / decreased sweating	Tender or bright erythema, patchy moist desquamation / moderate edema	Confluent, moist desquamation other than skin folds, pitting edema	Ulceration, hemorrhage, necrosis

0.75, 1, 1.25, 1.5, 1.75, 2, 2.5, and 3.0 Gy were delivered to the ten groups of the films. The calibration process was conducted at a depth of 10 cm at SSD=100 cm in a solid water-equivalent phantom (PTW, Germany). In order to obtain inherent optical density, the films were scanned by a Microtek scanner (Scan Maker 1000XL Pro: Microtek International Inc, Hsinchu, Taiwan) 24 hr prior and 48 hr following the irradiation.

Three 2 × 2.5 cm² pieces of GA Chromic films were placed on the skin of the patient chest. Two of the film pieces were placed at the medial and lateral region of the treatment field (2 cm from margin of field) and the third one at the center.

Calibration curve and corresponding equation fitted to our variables, absorbed dose (in Gy) and net optical density were used for interpretation of readings obtained from films which were placed at the three predefined locations.

Lymphocyte irradiation

Peripheral blood mononuclear cells (PBMCs) were separated from the 5 ml EDTA blood samples by density-gradient centrifugation using Ficoll (Cedar lane Lab, Canada) according to the manufacturer's instructions. PBMCs were washed twice with physiological phosphate buffer saline (PBS) and finally re-suspended in the 10 ml culture medium containing RPMI 1640 (GIBCO, Germany), 10% fetal bovine serum (FBS, Biosera, France), 100 Iu/ml penicillin, and 0.1 μg/ml streptomycin. Each sample was divided into three flasks including 1 and 2 Gy radiation and control group. Lymphocytes were irradiated by 6 MV X-rays to deliver 1 and 2 Gy *in-vitro* on ice.

Gene expression assay by real-time polymerase chain reaction (PCR)

Lymphocytes were collected 4 hr following to irradiation for ribonucleic acid (RNA) isolation from the cultures. The cells were washed with PBS, and then RNA was extracted by the TriPure reagent (Roche Applied Science, Germany) according to the manufacturer's recommendations. RNA sediments were dissolved in 20 μl Diethyl pyrocarbonate (DEPC)-treated RNase-free water and were assessed by electrophoresis on agarose gel. First-strand cDNA was synthesized from 1 μg of total RNA with oligo (dT) 18 primer using the RevertAid ™ First Strand cDNA Synthesis kit (Fermentas, Germany) in the same way as was described in our previous study (20). Gene expression assessments were performed on a StepOne (48‑well) real-time PCR system (Applied Biosystems) by SYBR® Premix Ex Taq™ (Takara, Japan) as described previously (20). Relative quantitative real-time PCR method was employed to assess *Bax* and *Bcl-2* gene expression levels. Beta‑2 Microglobulin (β2M) was used as a reference gene to normalize the quantity of the target genes .The sequences of primers are listed in Table 2.

Table 2. Primers used for apoptotic genes in SYBR green real time PCR

Gene	Sequence (5'-3')
β2M	Forward: GTATGCCTGCCGTGTGAAC
	Reverse: AACCTCCATGATGCTGCTTAC
Bcl‑2	Forward: TACTTAAAAAATACAACATCACAG
	Reverse: GGAACACTTGATTCTGGTG
Bax	Forward: GCTTCAGGGTTTCATCCAG
	Reverse: GGCGGCAATCATCCTCTG

Statistical analysis

The correlation between the variables was estimated by Pearson correlation coefficient. Statistical significance of the mean differences between the studied groups was evaluated by two-way ANOVA with correction for multiple comparisons via Tukey's *post hoc* test. Statistical calculations were performed with the GraphPad Prism, version 7.01. *P*-value less than 0.05 was considered statistically significant.

Results

Skin dose

To predict a possible relationship between surface dose and the skin reactions for individual patient, GAFChromic films were placed at three points on the chest wall skin.

The results obtained from the surface dosimetry and the Pearson's correlation analysis between these results and weekly acute skin reactions are presented in Table 3. As it is evident from Table 3, there is no significant relationship between skin dose and skin reactions.

Dose response curve for apoptotic genes

Apoptotic gene expression was measured based on *Bax*, *Bcl-2*, and *Bax/Bcl-2* ratio. In the *in-vitro* experiment, when lymphocytes were exposed to 1 and 2 Gy, *Bax* and *Bax/Bcl-2* ratio were increased with the dose (Figure 1; *P*<0.001 and *P*<0.0001, respectively by two-way ANOVA). Regression analysis showed that dose-response of both *Bax* and *Bax/Bcl-2* ratio groups was significant (*P*<0.001), and the mean slopes of the linear curves fittings were obtained 2.23±0.58 and 3.144±0.77, respectively. Therefore, *Bax/Bcl-2* ratio was determined as a biomarker of radiation response in the study.

Table 3. Results of Pearson's correlation analysis of surface dose at three locations and acute skin reactions of 10 patients in the five weeks

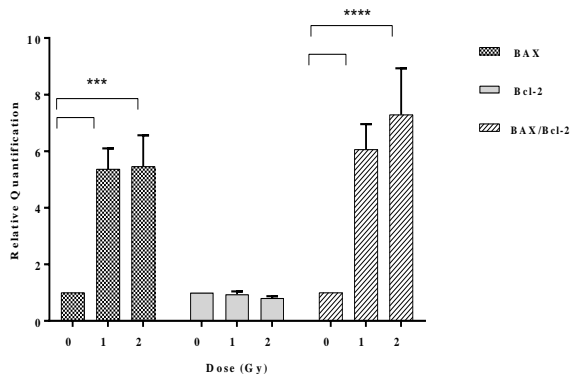|	Central	Medial	Lateral
Average dose:	1.739257	1.536981	1.385739
SD:	0.239389	0.326401	0.316626
Week 1	*P*= 0.9879	*P*= 0.6797	*P*= 0.8993
Week 2	*P*= 0.7932	*P*= 0.4794	*P*= 0.6742
Week 3	*P*= 0.2065	*P*= 0.0698	*P*= 0.0591
Week 4	*P*= 0.4575	*P*= 0.0612	*P*= 0.5052
Week 5	*P*= 0.2188	*P*= 0.0606	*P*= 0.1788

Figure 1. *Bax* expression, *Bcl-2* expression and *Bax/Bcl-2* ratio patterns revealed by relative quantitative

Effect of 0–2 Gy irradiation on *Bax* expression, *Bcl-2* expression and *Bax/Bcl-2* ratio in human peripheral blood lymphocytes after 4 hours. Each bar represents mean value for the ten patients and Error bars show standard Error of mean.

 ***represent *P-value*=0.001

****represent *P-value*=0.0001

Association between acute clinical radiation sensitivity and in-vitro induced apoptosis in BC patients treated with RT

The BC patients were subdivided according to the RTOG score for acute skin radiotoxicity (five weeks following RT). Based on this characterization, significant differences were found only for RTOG score in the fifth week versus the first four weeks (*P*<0.05). The *in-vitro* variation of *Bax/Bcl-2* ratio upon irradiation with 0–2 Gy was determined and the slope of the dose-response curve for *Bax/Bcl-2* ratio was calculated for every given individual. The correlation between slope of dose-response curve (as an *in-vitro* RS index) and acute skin reactions (as a clinical RS index) was examined by linear regression analysis. No significant relationship was detected in the first three weeks (Figures 2A-C). Interestingly, a significant correlation was found between dose-response curve and toxicity score four (Figure 2D) and five (Figure 2E) weeks following commencement of RT. The results were excluded for two patients (N-2 and N-4) who were outliers (beyond the 90th or 10th percentiles; Figure 3).
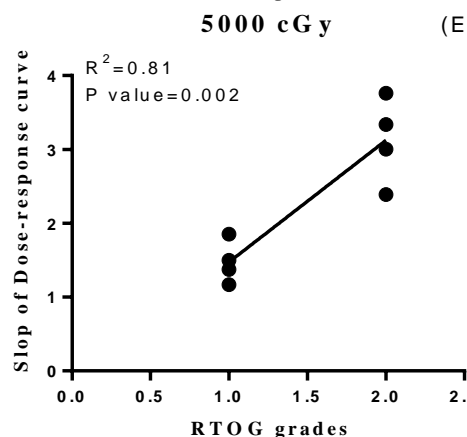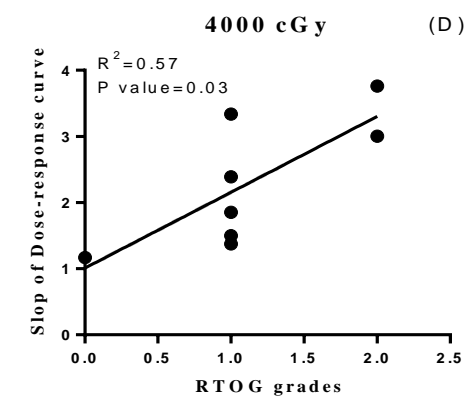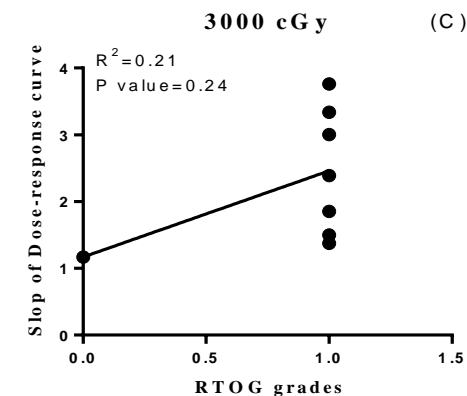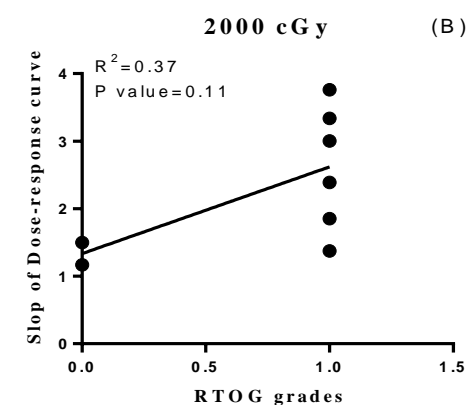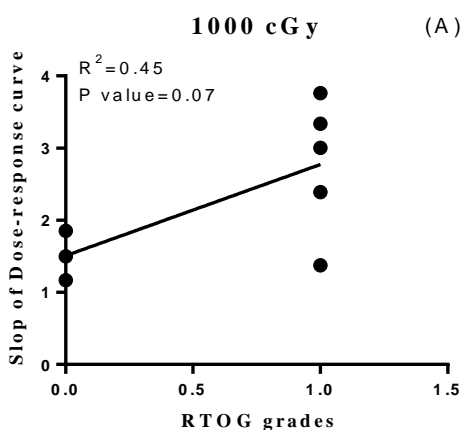

Figure 2. Simple linear regression lines, R-square and *P-values* showing the correlations between slop of dose-response curve and acute skin reactions. (A) One week (B) Two week (C) Three week (D) Four week (E) Five week treatment by 2 Gy per fraction
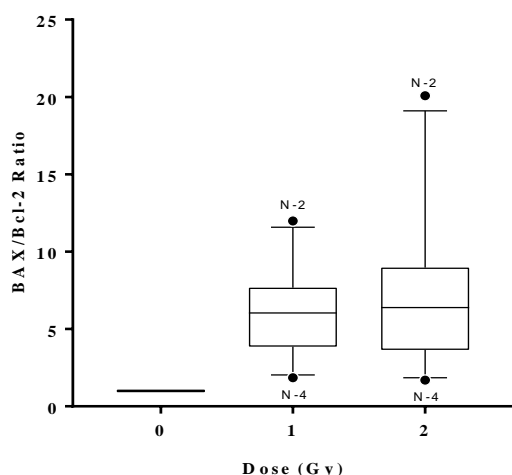
Figure 3. Distribution of *Bax/Bcl-2* Ratio in *in-vitro* irradiated PBMCs of 10 patients. the whiskers above and below the box represent the 90th and 10th percentiles, respectively. The points outside the whiskers are outliers beyond the 90th or 10th percentiles

Discussion

The aim of this study was to assess the relationship between gene expression level and acute clinical side effects in BC patients undergoing RT. The occurrence and severity of RT side effects were considered as RS indicators of the individual patient. It should be noted, acute skin reactions are not only influenced by genetic susceptibility to radiation, but also affected by the physical characteristics of radiation exposure including: intensity, conditions of RT, and other modifying factors. In this study, in order to verify this issue, surface dose and clinical radiation reactions were determined and no correlation between these variables were found (Table 3). In other words, our results has confirmed that skin reactions of the patients participating in this study are not due to physical characteristics of radiation and attributed only to inherent sensitivity to RT. The nature and causes of this phenomenon are still subject to debate and remains as a challenge to be solved. Quantification of different cell function such as cell survival, DNA damage, repair capacity, chromosome aberrations, cell death, and apoptosis can be an indicator of RS in a theoretical perspective. In the past two decades a number of researchers have referred to some markers of cell function that potentially can predict RS, but none have approached routine clinical practices (12, 15, 17, 30, 31). The results of previous studies have confirmed that apoptosis plays a critical role in tumor response to chemoradiotherapy (32, 33). The potential of the *Bcl-2* and *Bax* expression and apoptosis as predictive markers for RT response in cervical cancer was proposed by Qin *et al* (34). This

suggests that the differences between the individuals in RS may be due to changes in apoptosis-associated genes. The aim of this study was to assess apoptotic genes expression in order to validate these biomarkers as a prognostic marker of RT outcomes in BC patients. We have quantified *Bax* and *Bcl-2* expression levels in PBMC samples from 10 BC patients exposed to 1 and 2 Gy X-rays *in-vitro*. The measured expression of *Bax* and *Bax/Bcl-2* ratio increased significantly with dose. However, our data did not show any significant change in *Bcl-2* expression (Figure 1). Since the dose-response curve is unique for each patient, the slop of *Bax/Bcl-2* ratio was considered as an individually *in-vitro* RS index. Interestingly, the slop of dose-response curve and RTOG score is significantly correlated four and five weeks after commencement of the RT (as is shown in Figure 2). The significant relationship between *Bax/Bcl-2* ratio and toxicity score can be employed as a potential marker of RS prediction. These finding supports previous studies that reported the patients with Bcl-2- positive and Bax-negative (low Bax/Bcl-2 ratio) were not benefiting from RT and their tumors did not exhibit a probable susceptibility to apoptosis (35). Our findings are consistent with those of Yuan *et al.* who demonstrated the potential of apoptosis induction as a predictor of RS patients with cervical cancer (36). *Bcl-2* expression has been associated with recurrent localized prostate cancer after RT and increased level of *Bcl-2*, with low *Bax* levels correlated with high resistance to apoptosis in metastatic prostate carcinoma which corroborate the results of present study (30). Contrary to the present results, Harima (1998) did not found any relationship between response to RT of human cervical carcinoma patients and *Bax* and *Bcl-2* expression levels prior to RT (37). In conformity with the present results, previous studies have showed *Bax/Bcl-2* ratio as a predictive marker. Lee *et al.* used western blot analysis, and demonstrated that *Bax/Bcl-2* ratio reflected the cellular RS of some cell lines in pancreatic cancer cells (38). Similarly, an association was found between *Bax/Bcl-2* ratio and clinical response to chemoradiotherapy in bladder cancer patients based on immunohistochemistry (39). Previous studies have evaluated gene expression levels and revealed some genes can be used as an indicator to predict the RS. To help the readers with making better comparison, in Table 4, we compared the results of current study with the recent *in vitro* studies carried out on RS prediction by some gene expression.

Table 4. Comparison between current study and the recent *in-vitro* studies carried out on RS prediction by gene expression

Reference	Cell Type	Method	Gene(s) Investigated	Conclusion
Hernandez LAH *et al.* (40)	*PBMCs of BC patients	Microarray	*Microarray probe*	Significant associations between the gene expression profile and the development of acute and late toxicity in consecutive, unselected patients
Mayer *et al.* (41)	*PBMCs from head and neck and BC patients	Microarray	*Microarray probe*	A set of 67 radiation-induced genes was potentially capable to differentiate between radiosensitive and normal reacting patients
Torres-Roca *et al.* (42)	cancer cell lines of numerous types	real-time PCR	*RbAp48, RGS19 R5PIA Cox-2*	Gene expression profile could be used to predict SF2** as a dose response and RS index
Lu X-X *et al.* (43)	EC9706 esophageal cancer cells	real-time PCR	*MMP2 Bcl-2 Bax*	Downregulation of *Cox-2, MMP2* and *Bcl-2* expression followed by upregulation of Bax expression, are associated with increased RS
Mohammadi M *et al.* (44)	TE1, TE8 and TE11 esophageal cancer cell lines	real-time PCR	*Hdm2 P53*	Regulation of *Hdm2* and *P53* gene expression could distinguished radiosensitive compared to radioresistance cell lines that may occur due to apoptosis
Badie C *et al.* (45)	*PBMCs of BC patients	real-time PCR	*CDKN1A, GADD45A, CCNB1, and BBC3*	Post-irradiation expression response was significantly reduced for CDKN1A in severe reactors compared to normal
Wei *et al.* (46)	Peripheral blood plasma	real-time PCR	*miR-145*	Plasma *miR-145* is reduced in cervical cancer and is a novel candidate biomarker for diagnosing CC and predicting RS
Current study	*PBMCs of BC patients	real-time PCR	*Bax Bcl-2 Bax/Bcl-2 ratio*	Significant correlations between *Bax/Bcl-2* ratio determined before RT and clinical can be used as a potential test to identify radiosensitive individuals

*Peripheral blood mononuclear cells
**SF2=survival fraction at 2 Gy

Finally, *Bax/Bcl-2* ratio can be used as a marker to identify radiosensitive individuals. Nevertheless, it should be noted that two patients were excluded from analysis. In other words, the new proposed biomarker did not recognize the RS of these two patients. There are several possible explanations for this finding. Firstly, various pathways are involved in radiation response including apoptosis. Accordingly, a single path may be insufficient for developing a predicting method of radiation outcomes. This corroborates the ideas of Sarosiek (47), who suggested expression levels of anti-apoptotic or pro-apoptotic genes alone cannot determine sensitivity. Secondly, quantification of different endpoints can be used to predict intrinsic RS. Combination of Several endpoints and concurrently RS calculation may be useful to decrease false positive and false negative findings. In accordance with the present hypothesis, Kunogi *et al* found that combination of apoptosis and γH2AX foci can be used to predict tumor cell RS *in vitro* which is in good agreement with our conclusion (48). This finding has important implication for developing individual cancer treatment because it correctly predicts RS of the eight patients. However, more research on this topic with a large sample size needs to be undertaken before the relationship between *in-vitro* RS and RT outcomes in BC patients is more clearly understood.

Conclusion

Although RT techniques have advanced from early years of 20th century till today nevertheless advancement in several relevant branches of science such as biotechnology, immunology and genetic has paved the way to shift our effort toward a personalized treatment. The reality of personalized medicine based on biomolecular markers has not been fully accepted, but the early findings are promising. Several researchers have tried to develop an *in vitro* test to estimate the response to RT based on various cellular and molecular processes. To identify the role of apoptosis in RT outcomes, *Bax/Bcl-2* ratio in PBMCs may be suggested as a potential predictive marker for radiation-induced toxicity in BC patients.

Acknowledgment

Conflicts of interest

The authors declare that no conflict of interest exists.

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