

Expression of Bax Protein as A Prognosis Factor of Radiotherapy Response to Decreased Tumor Size in Stage IIB-IIIB Cervical Cancer

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ABSTRACT

Background: Cervical cancer plays a role as the leading cause of cancer death for women in developing countries. Radiation therapy kills cancer cells through double strand breaks and the apoptotic process. Bax protein is one of the regulators of apoptosis. This study analyzed the role of Bax expression as a prognostic factor in radiation therapy response in reducing tumor size in patients with advanced cervical cancer (IIB-IIIB).

Subjects and Method: This research is a retrospective cohort observational analytic study from January 2021 to April 2021 in the Department of Obstetrics and Gynecology in collaboration with the Department of Anatomical Pathology, Dr. Moewardi Hospital, Surakarta, Indonesia. This study involved 30 cervical cancer stage IIB-IIIB patients. The level of Bax expression was determined by immunohistochemical examination. ROC curve analysis was used to find cut-off points and evaluate the sensitivity and specificity of Bax in the prognosis of radiotherapy in patients with advanced cervical cancer (IIB-IIIB). Chi square test was used to determine the relationship between Bax expression and changes in tumor size in cervical cancer patients.

Results: The ROC curve analysis showed that the AUC Bax score in prognosis of radiation therapy was 0.575 (CI 95%= 0.32 to 0.83) and the cutoff point was 62.5%. Based on the analysis of the ROC curve, the Bax expression of 62.5% shows a sensitivity of 56.5% and a specificity of 71.4%. Analysis of the relationship between Bax protein expression on changes in tumor size using the Chi Square test showed a P value of 0.390 ($p > 0.05$).

Conclusion: The expression of Bax protein cannot play a role as a prognostic factor in the response of radiation therapy to the reduction in tumor size in stage IIB-IIIB uterine cervical cancer.

Keywords: bax, radiotherapy, cervical cancer.

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BACKGROUND

Cervical cancer is one of the most common cancers in women in the world. Based on

Globocan in December 2020 the incidence of uterine cervical cancer in the world was 604,127 cases and the mortality rate was

341,831 cases. Meanwhile, for data in Indonesia alone, the incidence of cervical cancer was 36,633 cases with a mortality rate of 21,003 cases (Sung et al., 2021).

Treatment for cervical cancer depends on the degree to which the disease progresses on diagnosis and locally available resources, and may involve radical hysterectomy or chemoradiation, or a combination of the two. Conservative surgical procedures and preserving fertility have become the standard of care for women with low-risk early-stage disease. Advances in radiotherapy technology, such as intensity-modulated radiotherapy, have resulted in less treatment-related toxicity for women with locally advanced disease (Cohen et al., 2019).

The main target of radiotherapy is to damage the DNA chain in cells which will result in the active apoptosis of cancer cells and stop cell proliferation through 2 main routes, namely the intrinsic and extrinsic pathways. One of these apoptotic pathways increases mitochondrial outer membrane permeabilization (MOMP). MOMP allows the release of proapoptotic factors such as cytochrome c and SMAC/ DIABLO from the mitochondria into the cytosol to activate the cascade cascade. MOMP is usually considered a point of no return on the apoptotic pathway. After MOMP, caspase activation often occurs within minutes, leading to cell death (Chipuk et al., 2010; Tait and Green, 2010).

The Bcl-2 protein family is one of the main regulators of the apoptotic process. It consists of two opposing protein groups: apoptosis antagonists (Bcl-2, Bcl-XL, Mcl-1) and apoptosis agonists (Bax, Bak, Bcl-Xs) (Naseri et al., 2015). In healthy cells, Bax and Bak move between the cytosol and the mitochondrial outer membrane (MOM) at varying rates. In apoptotic conditions, Bax and Bak will be activated and accumulate in MOM, where they will oligomerize and me-

diates MOMP to release proapoptotic factors such as cytochrome c. In addition, Bax/ Bak is regulated by antiapoptotic proteins and BH3-only proteins occur in MOM (Edlich et al., 2011; Schellenberg et al., 2013; Todt et al., 2015). The expression of Bax which is pro-apoptotic will help the apoptotic process and be prognostic in some epithelial cancers.

Based on these facts, it can be concluded that the presence of Bax protein has potential to be a prognostic protein of cancer. However, the role of Bax protein in predicting the radiation response in advanced uterine cervical cancer (stage IIB-IIIB) is still unknown. Therefore, this study assessed the expression of Bax protein as a prognostic factor for the success of radiation therapy.

SUBJECTS AND METHOD

1. Study Design

This research is a retrospective cohort observational analytic research from January to April 2021 in the Department of Obstetrics and Gynecology in collaboration with the Department of Anatomical Pathology dr. Moewardi Hospital, Surakarta, Indonesia.

2. Population and Sample

All samples/ subjects were a cervical cancer patient with cervical cancer stage IIB - IIIB who came to the Gynecology Oncology Clinic, or in the treatment room of Dr. Moewardi Hospital, Surakarta in the Department of Obstetrics and Gynecology dr. Moewardi Hospital, Surakarta, Indonesia from January to April 2021. The number of subjects taken 30 research subjects taken using the Rule of Thumb method (Murti, 2013).

3. Study Variable

Protein BCL2 Associated X (BAX) was considered as an independent variables whereas decrease in tumor volume/ res-

ponse therapy was considered as a dependent variables.

4. Operational Definition of Variables

The BAX protein was defined as an accumulation of the percentage of BAX protein expression shown by immunohistochemical staining of the entire field of view at 100x magnification.

Response therapy was defined as a decrease in percentage of tumor volume. The response to therapy is considered positive if there was a decrease in tumor volume $>70\%$ and a negative response to therapy was considered if there was a decrease in tumor volume $\leq 70\%$ (Udiyanto et al., 2020).

5. Study Instrument

Pretest and posttest examination of the size of the lesion clinically measured using GE Voluson S8 ultrasonography machine by the researcher itself. Examination of the BAX Protein biomarker used BAX immunohistochemical assay reagent and the results were observed using the Olympus CX22 Microscope in the Department of Anatomical Pathology by an Anatomical Pathologist.

The study subjects conducted pretest examination of measurements of lesions volume with ultrasound as well as Bax biomarker examination. Then all subjects were given treatment in the form of complete and sequential radiation administration. Furthermore, posttest examination is done 2 weeks to 4 weeks after radiation includes lesion measurement using ultrasound.

Immunohistochemical (IHC) analysis

All 30 patient cervical biopsy tissue blocks embedded in paraffin and fixed with formalin were cut to a thickness of 4 mm and placed on poly-L-lysine slides for incubation and deparaffinization. After that, the tissue was washed under running water and phosphate buffer saline 2 x 5 minutes. Retrieval (tris-EDTH, pH 9) was heated to a

temperature of 90 degrees Celsius to be washed with phosphate buffer saline 2 x 5 minutes and dripped with endogenous methanol peroxidase 3% H_2O_2 for 20 minutes. Subsequently, staining for Bax was carried out with reagents Bax obtained, and the tissue was incubated for 18 hours in the refrigerator. A hematoxylin counterstain is applied to the tissue and the tissue is covered with deckglass for further examination.

Evaluation of IHC results

The evaluation of the IHC results was observed using the Olympus CX22 Microscope in the Department of Anatomical Pathology by an Anatomical Pathologist. All 30 samples observed the total accumulation of Bax protein. The total accumulation of cells that are stained (immunoreactive) will be calculated in the entire field of view using a 100x magnification, then the total percentage is calculated to be used as a percentage of Bax protein expression.

Tumor Size Evaluation

The patient's tumor volume was calculated before and after therapy using transabdominal ultrasound examination. Therapeutic success was defined as the clinical condition after radiation therapy as indicated by a decrease in tumor volume in cm^3 which was evaluated clinically with a volume reduction of more than 70% relative to the tumor volume before radiation therapy was administered by ultrasound examination. If the volume reduction is less than 70% in cm^3 , radiation therapy is considered a failure.

6. Data Analysis

ROC analysis is used to obtain AUC values and cut off point expression of Bax proteins. Using ROC analysis results obtained sensitivity and specificity of the use of Bax expression in determining the radiotherapy prognosis of cervical cancer patients. The chi square test was used to determine the relationship between Bax at certain cut-off

points and tumor size changes in cervical cancer patients. The $P < 0.05$ is considered statistically significant. The entire analysis uses SPSS app for Windows version 25.

7. Research Ethics

This research obtained ethical approval from the Research Ethics Committee of RSUD Dr. Moewardi Surakarta with number: 69/II/HREC/2021.

RESULTS

This research was conducted from January to April 2021. 30 subjects from outpatients of Department of Gynaecology, Dr. Moewardi Hospital, Surakarta who met the criteria of the study subjects and were diagnosed with cervical cancer as well as received radiation therapy. Biopsies of cervical tissue were taken and immunohistochemical calculations were performed.

Figure 1 shows the results of Bax immunohistochemical examination in cervical biopsy of the study subject. Figure 1 describes the results of Bax immunohistoche-

mical examination with a total percentage of 95% at 100x magnification. The yellow arrow indicates that the Bax expression is positive in the cytoplasm.

The result of ROC curve coordinates is shown in Table 1 with Area under curve value of ROC curve expression Bax to radiation therapy response of cervical cancer stage IIB-IIIB is 0.575 (CI 95% 0.315 – 0.834). The percentage of Bax expression of 62.5% gives sensitivity of 56.5% and specificity of 71.4% is used as the cutoff point of the Bax expression.

In Table 2, chi square analysis shows significant number with p-value of 0.390 ($p > 0.05$) which shows that Bax expression above $\geq 62.5\%$ has relationship to tumor size change $> 70\%$. Risk analysis of variable Bax shows the OR= 3.25 (0.52 to 20.37) which indicates that the expression Bax $\geq 62.5\%$ has a probability (odds) of 3.25 times to experience a change in tumor size $> 70\%$ compared to the expression Bax $< 62.5\%$.

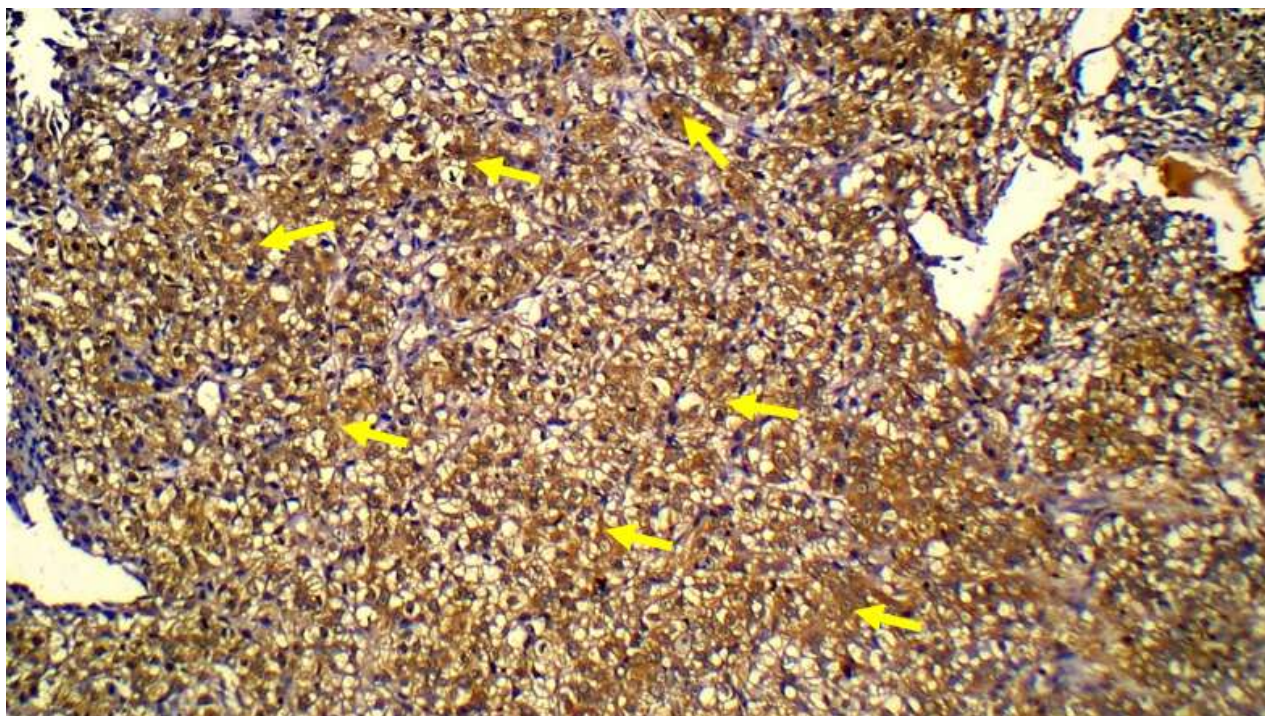
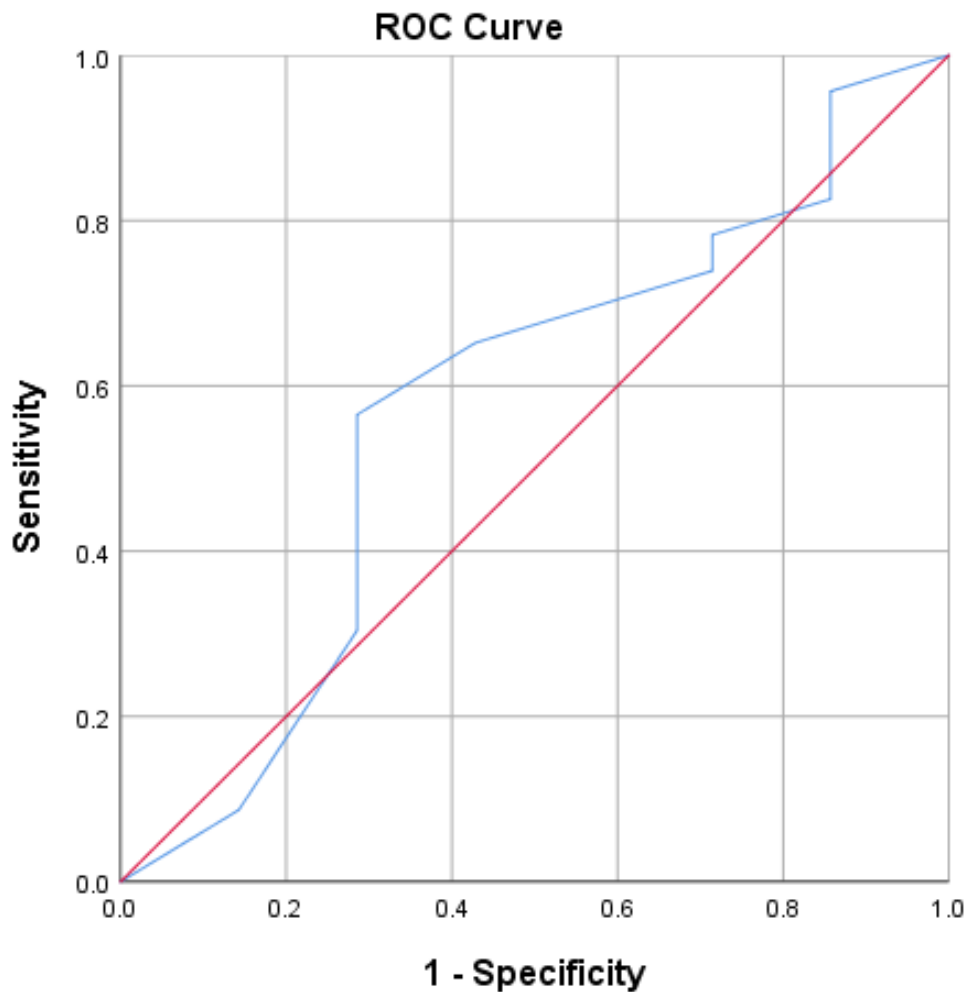


Figure 1. Bax immunohistochemical results of cervical cancer biopsy research subjects



Diagonal segments are produced by ties.

Figure 2. ROC curve to determine the cutoff point of Bax expression as well as the sensitivity and specificity of using Bax

Table 1. Percentage sensitivity and specificity of Bax expression at a particular cut off point

Percentage Bax Expression	Sensitivity	Specificity	AUC
4%	100.0%	0.0%	
17.5%	82.6%	14.3%	
22.5%	78.3%	28.6%	
45.0%	65.2%	57.1%	
62.5%	56.5%	71.4%	
67.5%	52.2%	71.4%	
85.0%	30.4%	71.4%	
96.0%	0.0%	100.0%	0.575

Table 2. Relationship of Bax protein expression to tumor size change based on ROC results

Bax Variable	Tumor Size Change (cm ³)		OR (95% CI)	P
	No Response (<70%)	Response (>70%)		
<62.5%	5 (16.7%)	10 (33.3%)	3.25 (0.52 – 20.37)	0.390
≥62.5%	2 (6.7%)	13 (43.3%)		

DISCUSSION

Apoptosis is an important programmed cell death pathway because it is indispensable for tissue homeostasis, embryonic development, and the body's immune system (Strasser et al., 2011; Czabotar et al., 2014). One of these apoptotic pathways increases mitochondrial outer membrane permeabilization (MOMP). MOMP allows the release of proapoptotic factors such as cytochrome c and SMAC/ DIABLO from the mitochondria into the cytosol to activate the cascade cascade. MOMP is usually considered a point of no return on the apoptotic pathway. After MOMP, caspase activation often occurs within minutes, leading to cell death (Chipuk et al., 2010; Tait and Green, 2010).

In vertebrae, the Bcl-2 protein family controls and regulates the intrinsic or mitochondrial apoptotic pathway. The Bcl-2 protein consists of various proteins with their respective functions such as the antiapoptotic Bcl-2 protein (Bcl-2, Bcl-xL, Bcl-w, Mcl1, and A1) as well as proapoptotic Bcl-2 proteins such as Bax and Bak which promoting MOMP directly. In order to regulate apoptosis, Bcl-2 proteins will interact with each other and produce complex interactions that will determine a cell to stay alive or die (Peña-Blanco and García-Sáez, 2018).

In healthy cells, Bax and Bak move between the cytosol and the mitochondrial outer membrane (MOM) at varying rates. In apoptotic conditions, Bax and Bak will be activated and accumulate in MOM, where they will oligomerize and mediate

MOMP to release proapoptotic factors such as cytochrome c. In addition, Bax/ Bak is regulated by antiapoptotic proteins and BH3-only proteins occur in MOM (Edlich et al., 2011; Schellenberg et al., 2013; Todt et al., 2015).

Under normal conditions, Bax is in the cytosol in an inactive form. During apoptosis, various conformational changes occur in Bax to allow permeabilization to MOM. After cytotoxic stress, Bax will accumulate in the mitochondria and become activated by their interaction with BH3-only proteins. The main action of Bax is the disruption of MOM which causes the release of proapoptotic factors to the cytosol. Proapoptosis such as cytochrome c and SMAC/ DIABLO will be released into the cytosol to activate the caspase cascade which in turn causes cell death (Peña-Blanco and García-Sáez, 2018).

Based on the chi square analysis comparing research subjects between subjects with Bax expression >62.5% and subjects with Bax expression <62.5%, there was an insignificant relationship ($p>0.05$) in subjects with Bax expression >62.5% in reducing tumor size >70%. compared with subjects with Bax expression <62.5%. In theory, Bax is a proapoptotic substance. In the event of apoptosis, it is hypothesized that there will be a smaller change in tumor volume if radiation therapy occurs. However, these results indicate an insignificant role for Bax in changes in tumor volume.

Based on the analysis of the ROC curve, the AUC number of Bax protein expres-

ssion for the prognosis of radiation therapy response was 0.58 (95% CI 0.32 to 0.83). The AUC result is an interpretation of the probability that a person with the disease will have a higher diagnostic test result than a healthy person. The AUC value can be interpreted like this: 90 - 100% = excellent; 80 - 90% = good; 70 - 80% = fair; 60 - 70% = bad; 50 - 60% = fail (Safari et al., 2016). Based on the results obtained, the Bax expression has an interpretation of AUC values that are between the numbers 50 - 60% so that the diagnostic test discrimination ability possessed by Bax expression cannot be used in determining the prognosis of the radiation therapy response to the success of reducing tumor size >70%. Using the ROC curve itself, the best cut off point of Bax expression was 62.5% with a sensitivity as high as 56.5% and a specificity of 71.4%. A sensitivity as high as 56.5% indicates the ability of Bax expression to predict unsuccessful radiation therapy responses (reduction in tumor size <70%) of 56.5%, while a specificity of 71.4% indicates the ability of Bax expression to predict successful radiation therapy responses (reduction in tumor size >70%).

The results obtained may be due to the influence of other apoptotic factors that cause the apoptotic ability of Bax to be not optimal. One of the possibilities is the predominance of the anti-apoptotic factor of the Bcl-2 protein family whose effect is greater than the ability of the apoptotic factor Bax which is responsible for the development of carcinogenesis in cervical cancer patients. In addition, this could be due to the alteration of other additional factors that have a crucial role in the progression of the cell cycle, individual telomerase ability, chromosomal stability and individual apoptotic ability, all of which are equally important in the transformation of cervical cancer severity (Ayatollahi et al.,

2014).

In Study Oh et al. (2010) in their research found that the E5 HPV16 protein was able to reduce the expression of the proapoptotic molecules Bax and Bak through various mechanisms, this led to an imbalance of antiapoptosis and proapoptosis in the protein of the Bcl-2 family. The Bax protein will normally translocate from the cytosol to the mitochondria and induce mitochondrial outer membrane permeabilization which results in the release of proapoptotic proteins such as cytochrome c and activation of the caspase protein family (Oh et al., 2010). The E5 protein of HPV16 in its findings stimulated Bax degradation via ubiquitin-proteasome-dependent degradation. Protein E5 of HPV16 increases ubiquitinated Bax thereby decreasing the life span of Bax protein. In addition, protein E5 of HPV16 is known to induce increased COX-2 expression and increased PGE2 secretion. Elevation of PGE2 was found to increase ubiquitination of Bax and decrease Bax expression and hydrogen peroxide-induced apoptosis through activation of EP2 and EP4 (Oh et al., 2009). Not only that, HPV E6 protein is also known to play an important antiapoptotic role in inhibiting Bax activity by inhibiting p53-dependent transcription of Bax through the mechanism of E6 protein in stimulating p53 degradation. This result causes E5 to also decrease release of cytochrome c to the cytosol and decrease Caspase-3 cleavage (Oh et al., 2010). All these findings suggest that there are various mechanisms that contribute to the progression and carcinogenesis of cervical cancer. This makes Bax itself unable to be used as a prognosis for the success of radiotherapy response because of the many other variables that affect the expression of Bax itself.

All in all, in this research we found that the expression of Bax protein does not

play a role as a good prognostic factor for radiotherapy response to the reduction in tumor size in stage IIB-IIIB uterine cervical cancer.

AUTHORS CONTRIBUTIONS

Conceptualization: Ahmad Fitriawan, Hermawan Udiyanto. Methodology: Hermawan Udiyanto. Validation: Affi Angelia Ratnasari, Wisnu Prabowo. Format analysis: Supriyadi Hari Respati. Writing-Original draft preparation: Ahmad Fitriawan, Wisnu Prabowo. Writing-Review and editing: Affi Angelia Ratnasari, Supriyadi Hari Respati

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CONFLICT OF INTEREST

There are no conflicts of interest.

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