

Research Report

Caspase 3 and Survivin Expressions as a Predictor of Response to Radiation Therapy in Advanced Stage Cervical Cancer

Ekspresi Caspase 3 dan Survivin sebagai Prediktor Respons Radiasi pada Kanker Serviks Stadium Lanjut

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Abstract

Objective: To identify prognostic factors of advanced stage cervical cancer which was treated by radiation therapy, so that they can be implemented to support treatment in order to increase the success of therapeutic response in advanced stage cancer.

Methods: Observational analytic historical cohort method study was conducted in Dr. Cipto Mangunkusumo General Hospital and 38 cases of stage IIB and IIIB cervical cancers receiving a complete radiation therapy were involved. Data were retrieved from medical records and cervical biopsies examination results. Paraffin blocks of cervical biopsies were examined histopathologically using HE staining followed by IHC. The expression of caspase 3 and survivin were then assessed by IRS scoring. Case group included 5 samples with negative therapeutic response, which means the therapeutic response other than complete response. Control group included 33 samples with positive therapeutic response which have complete response to therapy. The collected data were analyzed by univariate, bivariate, and multivariate analysis.

Results: Demographic and clinicohistopathologic data were not significantly related to the occurrence of negative response to radiation therapy. Negative cytoplasmic caspase 3 expression has a predictive value of 90.75% for the occurrence of a negative response to radiation therapy (RR=9.81, CI 95%=1.231-78.286, p=0.019). Positive cytoplasmic survivin has a predictive value of 86.75% for the occurrence of a negative response to radiation therapy (RR=6.55, CI 95%=2.659-16.119, p=0.000). Nuclear caspase 3 and survivin expressions have no significant relation to the occurrence of a negative response to radiation therapy. Clinical response to radiation is influenced by clinicohistopathologic factors, caspase 3, and survivin expression altogether.

Conclusion: Negative cytoplasmic caspase 3 expression and positive cytoplasmic survivin have predictive value for the risk of a negative response to radiation therapy to occur.

[Indones J Obstet Gynecol 2011; 35-3: 139-45]

Keywords: advanced stage cervical cancer, clinicohistopathology, caspase 3, survivin, response to radiation therapy.

Abstrak

Tujuan: Untuk mengidentifikasi faktor-faktor prognosis pada kanker serviks stadium lanjut yang dilakukan terapi radiasi sehingga dapat menunjang penatalaksanaan untuk meningkatkan keberhasilan respons terapi pada kanker stadium lanjut.

Metode: Penelitian dengan metode historikal kohort analitik observasional di RSCM terhadap 38 kasus kanker serviks stadium IIB dan IIIB yang telah dilakukan terapi radiasi lengkap. Data diambil dari rekam medis dan blok parafin biopsi serviks diperiksa histopatologinya dan dilakukan pewarnaan HE dan dilanjutkan dengan IHC untuk melihat ekspresi caspase 3 dan survivin dengan penilaian skor IRS. Kelompok kasus sebanyak 5 sampel adalah kelompok dengan respons terapi negatif yaitu respons stabil dan progresif. Kelompok kontrol sebanyak 33 sampel adalah kelompok dengan respons terapi positif yaitu kelompok respons komplit dan respons parsial. Data yang terkumpul dianalisis dengan analisis univariat, bivariat dan multivariat.

Hasil: Dari data demografi dan klinikohistopatologi tidak memiliki hubungan bermakna untuk terjadinya respons terapi radiasi negatif. Ekspresi caspase 3 sitoplasma negatif mempunyai nilai prediksi sebesar 90.75% untuk terjadinya respons terapi radiasi negatif (RR=9.81, IK 95%=1.231-78.286, p=0.019) dan survivin sitoplasma positif mempunyai nilai prediksi sebesar 86.75% untuk terjadinya respons terapi radiasi negatif (RR=6.55, IK 95%=2.659-16.119, p=0.000). Ekspresi caspase 3 inti dan survivin inti tidak memiliki hubungan bermakna untuk terjadinya respons terapi radiasi negatif. Respons klinik radiasi dipengaruhi oleh faktor-faktor klinikohistopatologi, ekspresi caspase 3 dan survivin secara bersama-sama.

Kesimpulan: Ekspresi caspase 3 sitoplasma negatif dan survivin sitoplasma positif mempunyai nilai prediksi untuk risiko terjadinya respons terapi radiasi negatif.

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Kata kunci: kanker serviks stadium lanjut, klinikopatologi, ekspresi caspase 3, ekspresi survivin, respons terapi radiasi

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INTRODUCTION

Cervical cancer is the most common cancer in women in developing countries.^{1,2} International Agency for Research of on Cancer (GLOBOCAN 2008) estimated that 529,000 new cases and 275,000 deaths from cervical cancer occur in the world every year and only 5% of women had been screened for cervical cancer.^{1,2} The 5-year survival rate of cervical cancer was

50% for stage I, 40% for stage II, 20% for stage III, and 0% for stage IV.³ Management of cervical cancer includes surgery, radiation, chemoradiation and chemotherapy, depending on the clinical stage, and this greatly affects the survival and the possibility of local control and loco-regional control.^{4,5} Radiation therapy and chemo-radiation are treatment options for advanced-stage cancer.^{5,6}

Up to now, it is known that the prognostic factors of cervical cancer are influenced by clinico-histopathologic factors.⁷ Tumor markers have been developed to determine prognostic factors of cervical cancer such as serum-associated antigens, angiogenesis factors, and apoptotic factors.^{8,9}

Dysregulation of apoptosis in cell plays an important role in the development and progression of malignancies in humans. Defects of apoptosis will lead to neoplastic processes through various mechanisms. Caspase is a family of protease which has an important role in pro-apoptotic process.^{10,11} Some studies which focused on apoptotic regulation-expression analysis showed the reduced levels of caspase in cervical cancer compare to the one in precancerous lesions.¹²⁻⁵

Beside the pro-apoptotic factors, there are also anti-apoptotic genes that are developed and known as inhibitors of apoptosis (IAPs).^{16,17} Survivin is an IAP which has 142 amino acids and molecular weight of 16.5 kDa. It contains a single baculovirus IAP repeat (BIR) which has capability to regulate cell proliferation and cell death. In the cell cycle BIR is expressed at the G2/M phases.¹⁸⁻²⁰ Survivin has an important role in tumorigenesis of colorectal cancer and virus-induced carcinogenesis.¹⁹ Suzuki et al, in their research found a correlation between survivin expression and locoregional control of cervical cancer which had under gone radiation therapy.²¹

Survivin in gynecologic cancer has not been much studied. It is expected that the new findings about the ratio of pro-apoptotic caspase 3 and anti-apoptotic survivin expressions can be used to predict the success of chemoradiation therapy for early and advanced stage cervical cancer. It is also expected that in the future the results of this study can be used as a reference for the study on gene targeted therapy for cancer. To identify prognostic factors of advanced stage cervical cancer which was treated by radiation therapy, so that they can be implemented to support treatment in order to increase the success of therapeutic response in advanced stage cancer is the aim of this study.

METHODS

The study was conducted at the Division of Gynecology Oncology, Department of Obstetrics and Gynecology, Faculty of Medicine, University of Indonesia, Dr. Cipto Mangunkusumo General Hospital Jakarta, and Department of Pathology Faculty of Medicine, University of Indonesia.

Population of this study was all advanced-stage cervical cancer patients (stage IIB, IIIA, and IIIB) who visited the Gynecology Oncology Clinic in Dr. Cipto Mangunkusumo Hospital Jakarta and had undergone radiation therapy from 2005 to 2010. Samples were taken from the case group and control group consecutively. Any subjects who met the inclusion criteria were included.

Secondary data was obtained from existing medical records of advanced-stage cervical cancer patients (stage IIB, IIIA, and IIIB) and was selected using the inclusion criterias stated. Course of the disease and radiation therapy accepted were observed.

Tissue that was taken by biopsy was preserved in 10% buffered formalin solution, and then sent to Department of Anatomic Pathology, Faculty of Medicine, University of Indonesia, where it, and embedded in paraffin. The paraffin block was processed by conventional histopathologic examination using Hematoxylin and Eosin staining (HE) and proceeded with the immunohistochemistry (IHC).

The primary caspase 3 antibody used in this study was rabbit monoclonal caspase 3 antibody (3CSP03): sc-56046 Santa Cruz Biotechnology, In. The primary survivin antibody used was mouse monoclonal survivin antibody Clone 12C4 reagent provided Code M3624 (Dako, Denmark). Detection instrument (detection kit) used was the Star Trek Universal Detection System HRP (Biocare Medical, LLC, Concord CA, USA) containing a Trekkie Universal Link; Trek avidin-HRP; Betazoid DAB chromogen; Betazoid DAB Substrate Buffer; Background Sniper.

Rabbit monoclonal caspase 3 antibody (3CSP03): sc-56 046 with a 1:600 dilution and Mouse monoclonal survivin antibody clone 12C4 with a 1:50 dilution were added into the paraffin block.

Both immunohistochemical staining were performed by incubation in a closed place at room temperature for 60 minutes and then rinsed with normal serum (PBS) 2 times, 5 minutes each. To bind the biotinylated secondary antibody using the immunoperoxidase method, a peroxidase (HRP) labeled streptavidin (Trekavidin-HRP) was added. Diaminobenzidine tetrahydrochloride /DAB (Betazoid DAB Chromogen) was added and given time to react for 2-5 minutes until it formed a brown chromogenic bond, followed with dehydration, cleaning, and fixation process into the object glass. Positive controls were taken from cervical carcinoma tissues with positive expressions of caspase 3 and survivin, while the negative controls were taken from the same cervical tissues which its primary antibody had been replaced with PBS.

Observations were made on 500 tumor cells from five different large fields of view (magnification of 400 times) randomly. Each field represented 100 tumor cells. Evaluation was carried out and analyzed by researchers and Anatomical Pathology specialists. The degree of positivity was determined by staining scoring using Immunoreactive Scoring System (IRS) modification, which was used by Remmele et al to analyze the expression of survivin.²² This scoring system can also be used to analyse caspase 3 expression. This scoring system was modified and determined by multiplying the intensity of staining with the percentages of positive cells. The percentage of positive cells was rated as follows: 0, 1-5% positive cells; 1, 5-25%; 2, 25-50%; and 3, > 50% positive cells. Staining intensity was scored as 1, weak; 2, moderate, and 3, intensive. Scores for percentage of positive cells and scores for expression intensities were multiplied to calculate an immunoreactive score (IRS), 0 = no staining; 1-2 = weak staining; 3-6 = moderate staining; > 7 = strong staining. It was classified as positive expression if the IRS > 3 for cytoplasmic survivin, cytoplasmic and nuclear caspase 3, and if the IRS > 1 for nuclear survivin.

After 3 months of complete radiation therapy, a clinical examination was performed to assess the re-

sponse to radiation therapy which was classified into complete, partial, stable, or progressive response.

RESULTS

Through the processes of selection according to inclusion and exclusion criterias, 38 cases of advanced stage cervical cancer that had been treated with radiation therapy and examined for caspase 3 and survivin expressions by the Department of Anatomical Pathology Faculty of Medicine, University of Indonesia, Jakarta, were taken. In this historical cohort study, the case group included 5 samples of patients with positive response to radiation, whereas the control group were 33 samples of patients with negative response to radiation therapy.

From the demographic and clinic-histopathologic data analysis, it was found that they have no significant relation to the occurrence of a negative response to radiation therapy.

From the analysis of cytoplasmic caspase 3 and response to therapy, it was obtained that the proportion of negative cytoplasmic caspase 3 was greater than positive caspase 3 in negative response to therapy (other than complete response), which was 36.36% compared to 3.70% with RR 9.81, CI 95% 1.231-78.286, and $p=0.019$ by Fisher's Exact test. Because negative survivin expression was not found in the unexposed group (complete response), a correction of number from 0 to 0.5 was done in the statistical calculation in order to do the bivariate test. The proportion of positive cytoplasmic survivin was

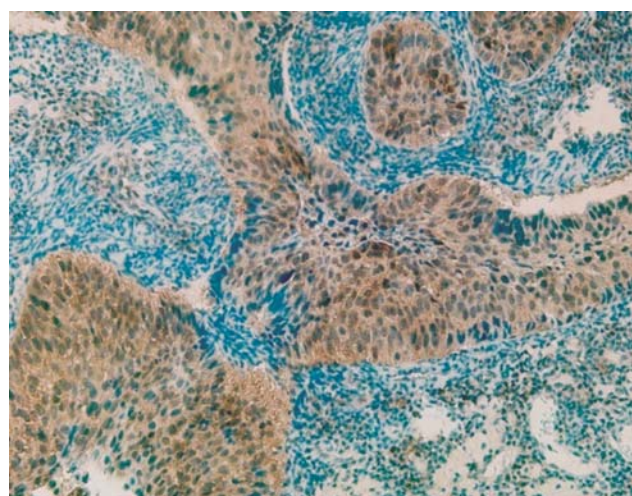
Table 1. Relations Between Patient's Characteristics and Response to Radiation Therapy

Characteristics	Response to Therapy				RR	CI 95%	p
	Negative		Positive				
	N	%	N	%			
Age							
> 50	1	7.14	13	92.86	0.43	0.53-3.46	0.63
≤ 50	4	16.67	20	83.33			
First Intercourse					2.88	0.55-15.15	0.32
< 19	2	8.00	23	92.00			
≥ 19	3	23.08	10	75.92			
Stage					0.54	0.10-2.75	0.59
IIIB	3	10.71	25	89.29			
IIB	2	20.00	8	80.00			
Volume					4.44	0.55-36.18	0.17
≥ 60	4	22.22	14	77.78			
< 60	1	5.00	19	95.00			
Histology					1.64	0.32-8.49	0.62
Adenocarcinoma*	2	18.18	9	81.82			
Carcinoma	3	11.11	24	88.89			
Differentiated					0.61	0.12-3.17	0.62
Moderate-Poor	3	11.11	24	88.89			
Well	2	18.18	9	81.82			
LVSI					0.61	0.08-4.89	1.00
Positive	1	9.09	10	90.91			
Negative	4	14.81	23	85.19			

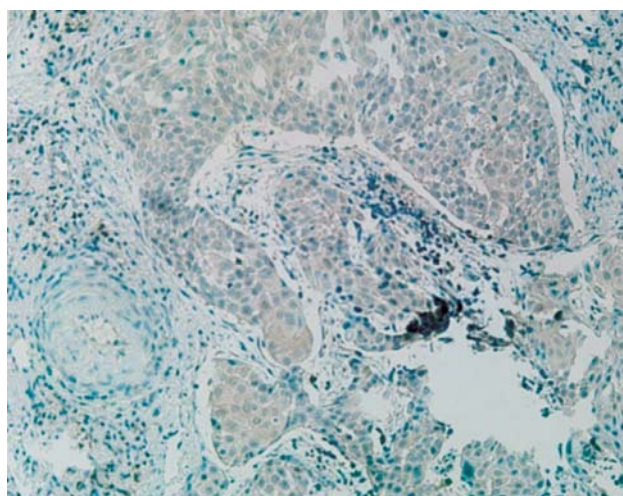
*Endometrioid Adenocarcinoma + Adenocarcinoma + Adenosquamous Carcinoma

Table 2. Relations Between Caspase 3 and Survivin Expressions and Response to Radiation Therapy

Expressions	Response to Therapy				RR	CI 95%	p
	Negative		Positive				
	N	%	N	%			
Cytoplasmic Caspase 3					9.81	1.23-78.29	0.02
Negative	4	36.36	7	63.64			
Positive	1	3.70	26	96.30			
Nuclear Caspase 3					1.85	0.35-9.85	0.64
Negative	3	17.65	14	82.35			
Positive	2	9.52	19	90.48			
Cytoplasmic Survivin					6.55	2.66-16.12	0.00
Positive	5 (4.5)	22.73	17 (17.5)	77.27			
Negative	0 (0.5)	0	16 (15.5)	100			
Nuclear Survivin					2.95	0.60-14.48	0.22
Positive	2	28.57	5	71.43			
Negative	3	9.68	28	90.32			

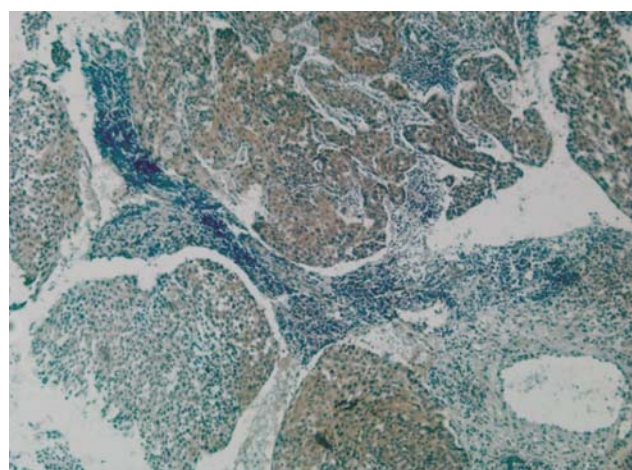


A. Positive

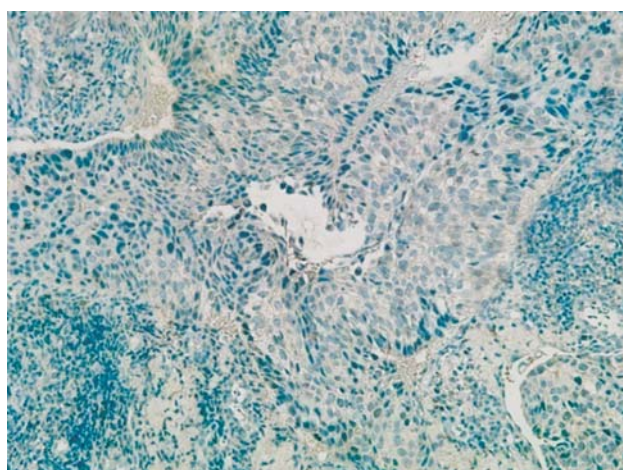


B. Negative

Figure 1. Cytoplasmic Caspase 3 Expression in slide with immunohistochemical staining with 400x enlargement.
A. Positive Expression. B. Negative Expression.



A. Positive



B. Negative

Figure 2. Cytoplasmic Survivin Expression in slide with immunohistochemical staining with 400x enlargement.
A. Positive Expression. B. Negative Expression

greater than negative cytoplasmic Survivin in negative response to therapy, which was 20.45% compared to 3.12% with RR 6.55, CI 95% 2.659-16.119, and $p = 0.000$ in Fisher's Exact test.

Nuclear caspase 3 and nuclear survivin did not have significant relations with negative response to therapy.

DISCUSSION

Modality of treatment for advanced cervical cancer (stage IIB and IIIB) is external radiation of whole pelvic followed by internal radiation, and in addition is concurrent chemotherapy with platinum base.⁵ Radiotherapy plays an important role in determining therapy for all stages of cancer.²³ It was believed that the combination of chemotherapy and radiation can control the disease locally, whereas chemotherapy alone controls the sub-clinical metastases that occur outside the radiation field.⁶

Tumor resistancy is a serious problem in the treatment of patients with neoplastic agents. Although it is still in controversy, the accumulation of experimental evidences showed that the initial damage caused by chemotherapeutic agents assembles into common apoptotic pathway. Upregulation of the protein inhibitor of apoptosis would be advantageous for tumor cells.²⁴ Several studies have found that in the tumor resistancy, there is enhancement of the expression of apoptosis inhibitor protein, especially survivin.²⁵

There was no difference in the risk of negative therapeutic response occurrence among various demographic data.

According to FIGO, one of the most important prognostic factors in cervical cancer is the clinical stage. Kleinberg et al, found that stage IB, IIA, and IIB have a significantly better survival rate compared to stage III or IVA treated by chemoradiation.²⁶ This study observed only stage IIB and IIIB. There were no significant differences for the risk of negative response occurrence with $p = 0.592$ between the two

were, but the proportion of negative prognosis of therapeutic response was greater in the stage IIIB group than in stage IIB, with a relative risk of 0.54 with CI 95% 0.104-2.754.

Based on ROC analysis, the cut-off point of tumor size was 60 cm³. The proportion of negative response to radiation therapy was higher in tumor size that greater than or equal to 60 cm³ (22.22%) than less of 60 cm³ (5.00%), with a relative risk of 4.44 CI 95% 0.546-36.176, although it was not statistically significant ($p = 0.170$). Assessment of therapeutic response through physical examination has limitations because of intratumor necrosis and fibrosis that occur in the cervix. CT scans and physical examination also has the limitations because they can not differentiate between the tumor residue from post therapy fibrosis and the tumor residue from post inflammatory fibrosis. This can only be differentiated by MRI.

Nakanishi et al stated that tumor size is a significant prognostic factor in stage IB cervical cancer with cut-off point 4 cm.²⁷ Huang et al also reported significant differences of response in 80 cases treated by radiation therapy, where the complete response (local control) occurred in the mean tumor volume of 66 cm³ (3.0 - 342.0) while negative response (local failure) occurred in the mean tumor volume of 129.5 cm³ (41.0 - 700.0) with the mean regression ratio for 0.6% and 19.4%.²⁸

The most common histology type of cervical cancer is squamous cell carcinoma. Based on FIGO, histology type can affect the prognosis. In several studies, adenocarcinoma and adenosquamous affect 5-year survival rate.²⁹ In this study, the most frequent type was squamous cell carcinoma (71.05%). The frequency of endometrioid adenocarcinoma, adenocarcinoma and adeno-squamous carcinoma was respectively 2.63%, 18.42% and 7.89%. There were no significant differences in the occurrence of a negative response to radiation therapy between the groups of squamous carcinoma and the groups of adenocarcinoma (adenocarcinoma, adeno-squamous, and endometrioid adenocarcinoma), with RR 1.64, CI 95% 0.315-8.487, $p = 0.615$.

Similar results were obtained by Long et al, squamous cell carcinoma 75%, adenocarcinoma 20%, and adenosquamous 2 - 3%. According to clinical histopathology analysis by Kleinberg et al, there were no significant differences between the histology of squamous cell carcinoma, adenocarcinoma and adenosquamous.²⁶ Peters et al in the Gynecologic Oncology Group (GOG) 109/Southwest Oncology Group (SWOG) 8797 found that patients with adenocarcinoma or adenosquamous treated with radiation alone had a poor progression-free survival rates. They also found that there was no difference in prognosis between radiation alone and chemoradiation therapy.³⁰

Other histopathologic factors showed no significant predictive value for the occurrence of a negative response to radiation therapy.

Caspase 3 and Survivin Expressions

Immunohistochemical examination showed that in advanced cancer, positive of caspase 3 were found more

frequently in the cytoplasm (71%) than in the nucleus (55.2%). Similarly, survivin were found more frequently in the cytoplasm (56.8%) than in the nucleus (5.26%). Similar result had been reported by several studies.³¹⁻³ Caspase 3 is one of the effector caspase activated by initiator caspase (caspase 8 and 9). Cytochrome C that is released from the intermembran chamber will bind the Apaf-1 and caspase 9. This process will activate the proteolytic caspase 3. SMAC/diablo will also be released from the mitochondria at the same time. It can be inverted that the process of apoptosis occurs in the cytoplasm.³⁴ While working as an inhibitor of Bax and Fas (signal of apoptosis), survivin also inhibits the process of caspase 3 and 7 (pro-apoptosis). All these processes also occur in the cytoplasm. It was relevant with this study, immunohistochemical staining of caspase 3 and survivin positive occurs more frequently in the cytoplasm.³⁵ Positive result in the nucleus occurs because the apoptotic process involves the cell cycle.

Cytoplasmic caspase 3 has strong relation with the response to radiation therapy. The response is influenced by the expression of caspase which increase the apoptosis process. Characteristics of apoptosis in stereotyped morphologic picture are chromatin condensation, nucleus fragmentation, and membrane closure of apoptotic body. The changes of this morphology are executed by the caspase family. Caspase 3 as an executioner caspase activates this apoptotic function. Radiation exposure will induce damage of DNA via extrinsic pathway by forming a caspase arcade and will start the process of apoptosis. FADD stimulation will alter a pro-caspase 8 to caspase 8 which will stimulate apoptosis. Caspase 8 and 9 alone will stimulate the pro-caspase 3 to become caspase 3 and then caspase 3 will further regulate apoptosis.³⁶⁻⁷ Cell death occurs during the transition from metaphase to anaphase is characterised by the activation of caspase 2 as the initial activation of DNA damage response and by the release of cell death effectors such as caspase 9 and 3 as well as an activator of cytochrome C. High expression of caspase 3 in the cytoplasm activates apoptosis. Cells that fail to execute the apoptotic program with the failure of mitosis will have asymmetric division in the next cell division and will become aneuploid cells. This will non-activates apoptotic program and causes chromosomal disorders.³⁸

In this study, the cut-off point of ROC analysis was > 3 . We found that the specificity of positive cytoplasmic caspase 3 was 80% while the sensitivity was 62.50% (ROC area = 0.7542). Of the 38 samples, we found that there was a significant negative relation between cytoplasmic caspase 3 and response to therapy with a value of $p = 0.031$. Negative expression of cytoplasm caspase 3 increases the risk of a negative response to radiation therapy with RR 4.09 and CI 95% 1.174-14.249. There is no significant relation between expression of nuclear caspase 3 and radiation response with $p = 0.426$, although the proportion of negative nuclear caspase 3 (29.41%) was greater than positive nuclear caspase 3 (14.29%) for the occurrence of negative responses with a relative risk of 95% CI 2058 0572 -7414.

The experiment by Huang et al on experimental animal which were mice HNSSC (head neck

squamous cell carcinoma) and mice breast cancer treated by radiation therapy shows that positive caspase 3 has better survival rate compared with the negative caspase 3 ($p = 0.0114$ and $p = 0.0006$).³⁹ In the study by Cheung et al, the expression of caspase 3 was reported. In the study by Shi et al to be related to cervical cancer in which reduced expression of caspase 3 inhibits apoptosis process.³¹

Survivin protein serves as an inhibitor of caspase activation that plays an important role in mitosis and negative regulation of apoptosis. The survivin expression is also increased by cell cycle regulation and is only expressed in the G2-M phase. The location of survivin is at the mitotic spindle that interacts with tubulin during mitosis.⁴⁰ Tamm et al in their study also found that survivin blocked apoptosis directly in 293 cells which had been transfected by over-expression of caspase 3 and 7 and plasmids encoding survivin. It was showed that survivin inhibits second process of caspase in the active form only. Survivin might only prevent the breakage of amplification activation cascade which decreased apoptosis process.³⁵ Survivin was thought to have a relation with radiation resistancy and hypoxia, so that over-expression of survivin causes resistance to therapy and poor prognosis.¹⁷ Bache in his study found that over-expression of survivin significantly decreased the 5-year overall survival with odds ratio 3.3 ($p = 0.02$) in bivariate analysis and 3.2 ($p = 0.03$) in univariate and Cox regression multivariate analysis.⁴¹ In the study by Shi et al, survivin is an independent prognostic indicator that affected survival rates for cervical cancer. There was shorter survival rates in groups with positive survivin expression ($p < 0.05$).³¹

Of the 38 samples, we found that cytoplasmic survivin expression is more positive than the nuclear survivin, which were 57.89% (22/38) and 18.42% (7/38). Suzuki et al found similar results which were positive cytoplasmic survivin 47% and nuclear survivin only 14%.²¹ We found that there was a significant positive relation between cytoplasmic survivin and response to therapy with a value of $p = 0.031$. Positive expression of cytoplasm survivin increases the risk of a negative response to radiation therapy with RR 6.55, CI 95% 2.659-16.119, and $p = 0.000$ in Fisher's Exact test.

It can be concluded that cytoplasmic caspase 3 and cytoplasmic survivin has a predictive value for the response to radiation therapy by using score > 3 , whereas nuclear caspase 3 and nuclear survivin can not be used as a predictor because caspase 3 is not activated in the nucleus but for the mitochondrial membrane. Survivin is expressed in the phase G2 and M of cell cycle and is located at the mitotic spindles so it should be expressed in the nucleus, but in our study it was not significantly related.

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