

RESEARCH ARTICLE

Survivin and Telomerase as Radiotherapeutic Response Predictors of Subjects with Stage IIB Cervical Squamous Cell Carcinoma

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Abstract

BACKGROUND: Cervical cancer is one of the most prevalent cancers in women. Even with similar clinicopathologic features, radiotherapy outcomes are still vary among patients. This research was conducted to measure radiotherapy responses on cervical cancer patients by using Survivin, Telomerase and Cytochrome C.

METHODS: Subjects who matched the criteria were selected and requested to fill questionnaires. Subjects were then evaluated with magnetic resonance imaging (MRI) pre- and post-3D conformal radiotherapy. Histopathological study was conducted using resected tumors to determine the differentiation type. Enzyme-linked Immunosorbent Assays for detection of Survivin, Telomerase and Cytochrome C was performed using the resected tumors as well.

RESULTS: There were 76 patients in this study. Mean of age was 50 and diameter of tumor size was 5.35 cm. Mean levels of Survivin, Telomerase and Cytochrome C were

632.82 pg/mL, 5.59 pg/mL and 204.75 ng/mL, respectively. There were significant correlations between radiotherapy response and Survivin ($p=0.041$) or Telomerase ($p=0.022$). Subjects with lower Survivin level (<932 pg/mL) had higher 1-year survival rate (63%) than subjects with higher Survivin level (50%). Similar results were obtained for subjects with lower Telomerase level (<5.75 pg/mL), who had higher 1-year survival rate (60%) than subjects with higher Telomerase level (43%).

CONCLUSION: Since radiotherapy response is significantly correlated with Survivin and Telomerase levels and subjects with lower Survivin or Telomerase level have higher 1-year survival rate, it can be suggested that Survivin and Telomerase could be potential predictors of radiotherapeutic response for subjects with stage IIB cervical squamous cell carcinoma.

KEYWORDS: cervical cancer, radiotherapy Survivin, Telomerase, Cytochrome C

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Introduction

Cervical cancer is one of the most common cancers in women. Human Papilloma Virus (HPV) is closely related to the occurrence of cervical cancer.(1-4) The incidence of cervical cancer in Indonesia is 100 among 100.000 women. The mortality rate of patients with cervical cancer is still high

(90%), since the patients seek for help in the advanced stage of disease.(5) Nearly 70% of patients with cervical cancer in Dr. Cipto Mangunkusumo Hospital Jakarta were in the stage of IIB to IVB. Therefore, the outcome of treatments were still far from satisfaction.(6) Radiotherapy and palliative chemotherapy are the treatments of choice for cervical cancer stage IIB to IVB.(7-9) However, there are failures in radiotherapy, 10% at stage IIB, 42% at stage III and 74% at

stage IVA.(10) Success in cervical cancer therapy depends on clinicopathologic features, such as the cancer stage, tumor size, histopathological type, differentiation level and lymphocyte reaction.(5) However, radiotherapy outcomes in similar clinicopathologic features are not always alike.

Various tumor markers were studied to determine prognostic factor, such as serum associated antigen, angiogenic factor and apoptotic factor. Angiogenic factor was reported to have a role in early stadium, meanwhile apoptotic factor in both early and late stadiums.(11,12) Therefore, apoptotic factor such as Cytochrome C is important to be investigated. Cytochrome C, which is produced by mitochondria, comprises 104 amino acids and plays an important role in cellular development or death. (13) Cytochrome C plays a role in the intrinsic pathway type 2 via the mitochondrial electron transport system. The role of Cytochrome C has been reported in various humans cancers, including thyroid (14), lung (15), colon (16), prostate (17), breast (18,19), ameloblastoma (20), ovarian (21) and cervical cancers (22,23).

Despite Cytochrome C, Survivin also plays an important role in the apoptotic pathway. Survivin is a member of the inhibitor of apoptosis (IAP) gene family, comprises 142 amino acids. Survivin has the capability to control cell proliferation and apoptosis.(24,25) Level of Survivin is very low in healthy tissue, but it is increasing in tumor tissue.(24,26) Survivin overexpression is currently used as prognostic factor in the brain (27), breast (28), rectal (29) and bladder (30) cancers. In addition, level of Survivin has been used as a predictor for treatment outcome. Based on cumulative incidences in five years, the cancer recurrence is greater in patients with higher level of Survivin.(29)

Telomerase is a ribonucleoprotein that adds a species-dependent telomere repeat sequence to the 3' end of telomeres. By preventing DNA from shortening with each cell cycle, Telomerase is able to inhibit cellular senescence and apoptosis.(31) Telomerase activity was detected in 96.5% of cervical tumor samples and in 68.7% of premalignant cervical scrapings but it was not detected in control hysterectomy samples nor in cervical scrapings of normal healthy control. Therefore the absence of Telomerase activity in cervical scrapes of healthy women indicated the potential of Telomerase as a screening marker for early diagnosis of cervical cancer.(32) Since Cytochrome C, Survivin and Telomerase are important biomarkers for cervical cancers, therefore the present study was conducted to investigate the correlation of these biomarkers with the outcome of radiotherapy in the advanced stage of cervical cancer.

Methods

Subject Recruitment and Selection

A prospective study with nested case-control method was conducted to see the role of Survivin, Telomerase and Cytochrome C as markers of cervical cancer radiotherapy outcomes in addition to clinicopathologic features. The study was conducted in Dr. Cipto Mangunkusumo National Central General Hospital, Jakarta, from January 2016 to May 2017. Subjects were recruited and selected based on the inclusion criteria of having untreated cervical cancer with the extension into the pelvic sidewall, hydronephrosis or non-functioning kidney with histopathologic type of squamous cell carcinoma (stage IIIB according to *Fédération Internationale de Gynécologie et d'Obstétrique* (FIGO) staging of cervical cancer). Meanwhile the exclusion criteria were subjects diagnosed with Human Immunodeficiency Virus (HIV), severe systemic diseases, heart disease, sclerosis and wound healing. Prior to recruitment and selection, all subjects had agreed to participate in the study and signed the informed consent. The study protocol was approved by the Research Ethics Committee, Faculty of Medicine, Universitas Indonesia (Ethical clearance No. 446/UN2.F1/ETIK/2015).

Radiotherapy

Subjects who matched the criteria were evaluated with magnetic resonance imaging (MRI) before starting the radiotherapy. Radiotherapy was done using 3D conformal radiotherapy. The total dosage were 70 Gy, consist of external radiation for whole pelvis as primary therapy at a dose of 45-50 Gy divided into 1.8-2 Gy each fraction, 5 fraction per week (total 25-28 fractions), followed by Ir192 afterloading high-dose-rate (3x7 Gy) intracaviter brachytherapy. After 2-3 months, the therapy response was re-evaluated with MRI.

Radiotherapy responses were divided into good and poor response. Good response subjects were those who had complete response (no lesion, no new lesion, and no related symptom) or partial response ($\geq 30\%$ reduction of lesion size, no new lesion, no progressive disease). Poor response group was further divided into progressive response or no response. The definition of progressive response was $>20\%$ increase of the lesion size, growth of a new lesion, or the deterioration of the disease. No response subjects were those who could not be qualified as complete, partial, or progressive response.

Histopathology

Sections from the resected tumors were prepared and stained with hematoxylin and eosin. The sections were then analyzed by a pathologist to determine the differentiation type (well differentiated, moderately differentiated, poorly differentiated, and undifferentiated), based on The World Health Organization (WHO) classification.

Enzyme-linked Immunosorbent Assay (ELISA) for Survivin, Telomerase and Cytochrome C

ELISA for Survivin, Telomerase and Cytochrome C were performed using Human Surv (Survivin) ELISA Kit (Catalog No.: E-EL-H1584, Elabscience, Houston, USA), Human TE (Telomerase) ELISA Kit (Catalog No.: E-EL-H0164, Elabscience) and Human Cyt-C (Cytochrome C) ELISA Kit (Catalog No.: E-EL-H0056, Elabscience). Basically, each micro ELISA plate provided was pre-coated with an antibody specific to human Survivin, Telomerase or Cytochrome C. Standards or the resected tumor samples were added to the micro ELISA plate wells and combined with the specific antibodies. Then an Avidin-Horseradish Peroxidase (HRP)-conjugated detection antibody specific for human Survivin, Telomerase or Cytochrome C was added to each micro plate well and incubated. After washing, the substrate solution was added to each well. Only wells containing human Survivin, Telomerase or Cytochrome C appeared in blue color. The enzyme-substrate reaction was terminated by the addition of stop solution and the color turned yellow. The optical density (OD) was measured spectrophotometrically at a wavelength of 450 nm \pm 2 nm. The OD value was proportional to the concentration of human Survivin, Telomerase or Cytochrome C. Concentration of human Survivin, Telomerase or Cytochrome C in the samples was calculated by comparing the OD of the samples to the standard curve.

Statistical Analysis

Collected data were analyzed using SPSS for Macintosh ver. 20 (IBM Corp., Armonk, NY, USA). Bivariate analysis to assess relation of Survivin, Telomerase, and Cytochrome C activity on radiotherapy response was done by determining the value of Survivin, Telomerase, and Cytochrome C activity limit considered significant using Receiver Operating Characteristic (ROC). Furthermore, bivariate analysis was done to find the correlation between variables using chi-square method. If the requirement of chi-square test was not fulfilled, Fisher test would be done to evaluate the correlation between variables.

Results

A total of 76 subjects who met the criteria was further analyzed. Subject characteristics are shown in Table 1. Bivariate analyses of relation between subject characteristics and radiotherapy response were performed. There was no significant correlation between radiotherapy response and age ($p=0.07$), parity ($p=0.22$), first sexual intercourse age ($p=0.43$), diameter of tumor ($p=0.32$) and differentiation type ($p=0.08$).

Mean levels of Survivin, Telomerase and Cytochrome C were 632.82 pg/mL, 5.59 pg/mL and 204.75 ng/mL, respectively. Survivin, Telomerase and Cytochrome C levels were categorized using ROC curve analysis. Cut off limit of Survivin was set to 932 pg/mL, considering likelihood ratio (LR)+ value of 2.54, sensitivity 37.90%, specificity 85.10%, and accuracy of 57.90% (Figure 1A). Cutoff limit of Telomerase was set to 5.75 pg/mL, considering LR+ value of 2.26, sensitivity 46.80%, specificity 79.30%, and accuracy of 63.05% (Figure 1B). Meanwhile, cut off limit of Cytochrome C was set to 122.92 ng/mL, considering LR+ value of 1.09, sensitivity 78.70%, specificity 27.60%, and accuracy of 53.15% (Figure 1C).

Based on chi-square analysis, there were significant correlation between radiotherapy response and Survivin ($p=0.041$) or Telomerase ($p=0.022$). Relative risk of this study was 1.833 (1.067-3.150, CI 95%) for Survivin (Table 2) and 2.236 (1.038-4.820, CI 95%) for Telomerase (Table

Table 1. Subjects characteristics (n=76).

Characteristics	
Mean of age (years old)	50.29
First sexual intercourse	
<20 years old	33 (43.4%)
\geq 20 years old	43 (56.6%)
Median of parity	3 (0-8)
Diameter of tumor (cm)	5.35 (1.9 – 15.0)
Differentiation type*	
well and moderately	19 (25.0%)
poor and undifferentiated	57 (75.0%)
Radiotherapy response	
positive	49 (61.8%)
negative	27 (38.2%)

*Based on WHO

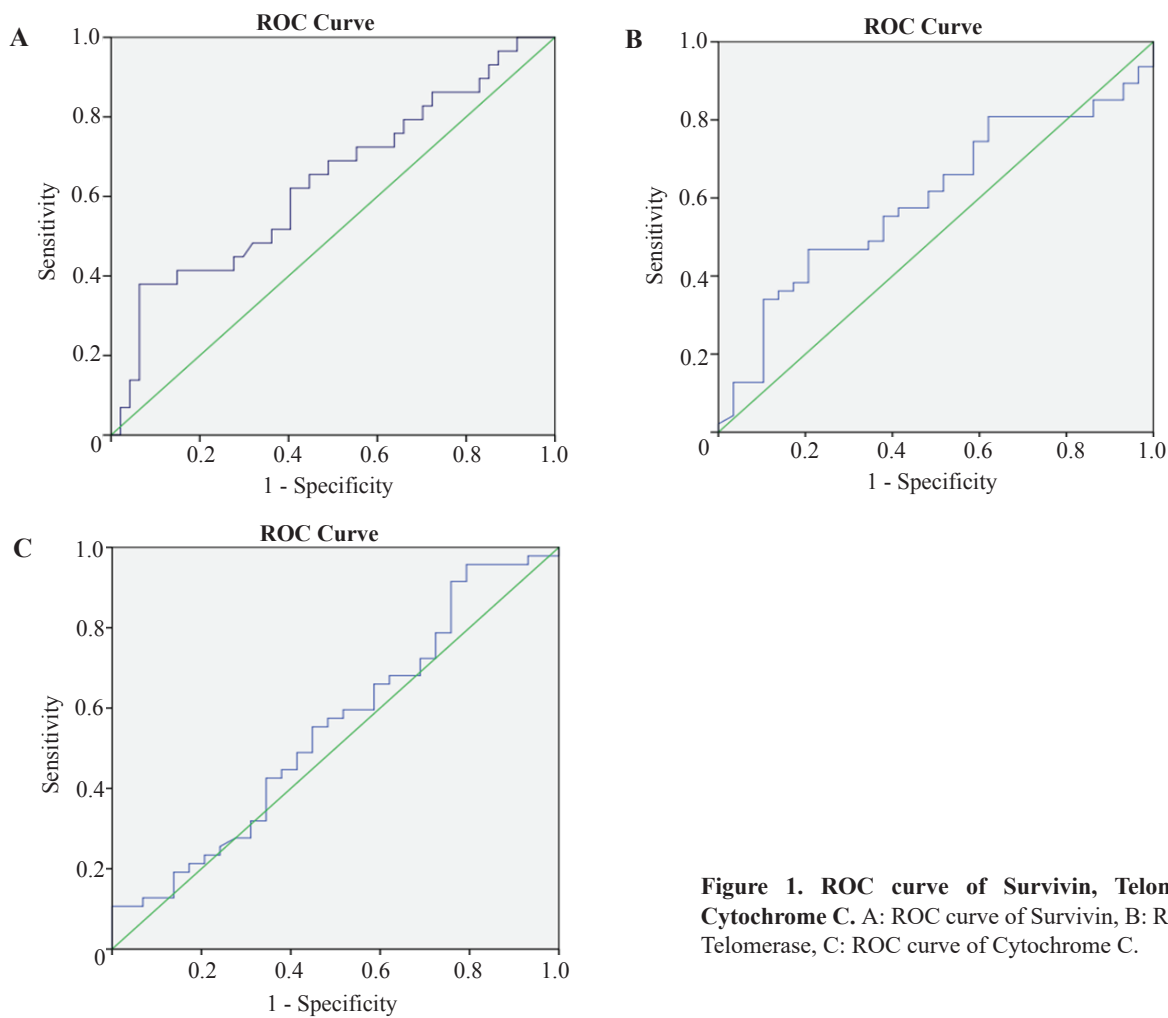


Figure 1. ROC curve of Survivin, Telomerase and Cytochrome C. A: ROC curve of Survivin, B: ROC curve of Telomerase, C: ROC curve of Cytochrome C.

3). However, there was no significant correlation between radiotherapy response and Cytochrome C ($p=0.530$).

Kaplan-Meier study was also done in this study to compare the subject survival rate of stage IIIB cervical squamous cell carcinoma with Survivin (Figure 2) and Telomerase (Figure 3). Subjects with lower Survivin level (<932 pg/mL) had higher 1-year survival rate (63%) than subjects with higher Survivin level (50%). Similar results were obtained for subjects with lower Telomerase level (<5.75 pg/mL), who had higher 1-year survival rate (60%) than subjects with higher Telomerase level (43%).

Discussion

From 2016 to 2017, most of patients with stage IIIB cervical squamous cell carcinoma in Dr. Cipto Mangunkusumo National Central General Hospital were 50 years old and had 3 children. This data is similar to a previously reported study which showed that the risk of having cervical cancer was higher in patients age of 50 years or more with odd ratio of 2.53.(7) However, our results showed no significant correlation between age, first sexual intercourse age, and

Table 2. Bivariate analysis between Survivin level and radiotherapy response.

Survivin Level (pg/mL)	Response		Total	p-value	Relative Risk
	Poor	Good			
≥932	11 (57.9%)	8 (42.1%)	19 (25.0%)	0.041*	1.833 (1.067–3.150, CI 95%)
<932	18 (31.6%)	39 (68.4%)	57 (75.0%)		
Total	29 (38.2%)	47 (61.8%)	76 (100.0%)		

*Tested with chi-square.

Table 3. Bivariate analysis between Telomerase level and radiotherapy response.

Telomerase Level (pg/mL)	Response		Total	p-value	Relative Risk
	Poor	Good			
≥5.75	23 (47.9%)	25 (52.1%)	48 (63.2%)	0.022*	2.236 (1.038–4.820, CI 95%)
<5.75	6 (21.4%)	22 (78.6%)	28 (36.8%)		
Total	29 (38.2%)	47 (61.8%)	76 (100.0%)		

*Tested with chi-square.

parity with radiotherapy outcome in stage IIIB cervical squamous cell carcinoma patients. Previous study also reported no significant correlation in patient age, overall survival, and relapse free survival.(8)

Our results showed that subjects with ≥ 932 pg/mL Survivin had significant lower 1-year-survival after radiotherapy. This is in accordance with another report that Survivin overexpression is correlated significantly with lower complete remission.(33) Overexpression of Survivin has a poor prognosis and high resistance to therapy. Survivin inhibits both extrinsic and intrinsic apoptotic pathways.(24) Survivin mutants (Thr34-Ala, Ser81Ala, Cys84Ala,) have more potential to inhibit the growth of hepatocellular cancer compared to the use of either one mutant alone.(34-37)

Subjects with high level of Telomerase (≥ 5.75 pg/mL) had significant poor response to radiotherapy, suggesting

that low level of Telomerase had a better prognosis of radiotherapy responses. Similar studies showed that low level of Telomerase had a better radiotherapy response. Another study reported that Telomerase had roles in tumor development and induction of chromosomal instability, therefore Telomerase activity can serve as a tumor marker.(38) In the contrary, the absence of proliferation was observed in human cells due to lack of Telomerase. Lack of Telomerase will limit tumor cell growth that duplicate continuously. Thus Telomerase inhibitor is a promising factor in eliminating cancer cells.(39)

Level of Cytochrome C was not correlated significantly with the radiotherapy response and 1-year-survival. Low level of Cytochrome C in the cytoplasm are assumed due to Cytochrome C was reserved in the mitochondria and not been released into the cytoplasm. Level of Cytochrome C

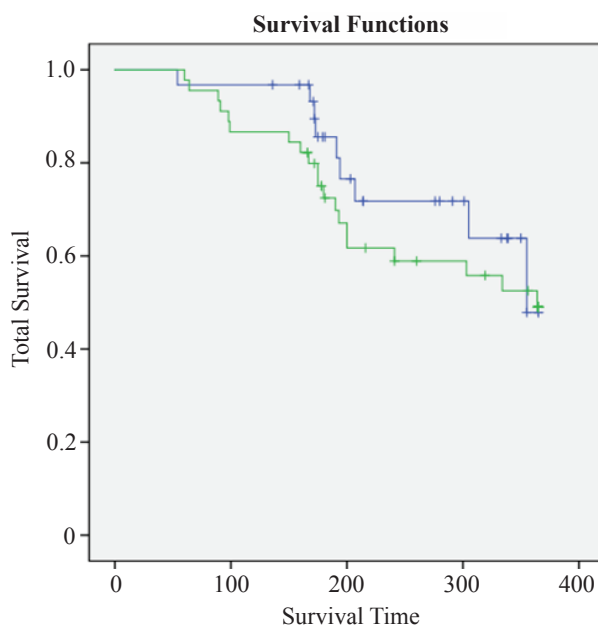


Figure 2. Kaplan Meier graph of 1-year-survival subjects with high and low Survivin level. Blue line: subjects with lower level of Survivin; Green line: subjects with higher level of Survivin.

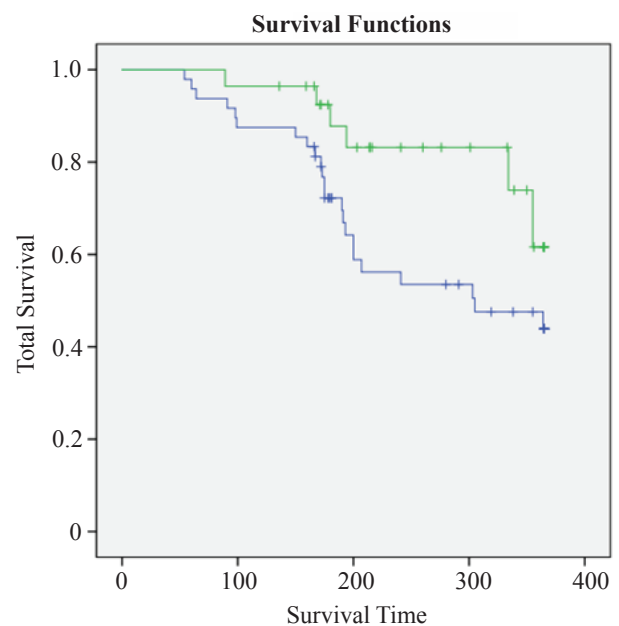


Figure 3. Kaplan Meier graph of 1-year-survival subjects with high and low Telomerase level. Blue line: subjects with higher level of Telomerase; Green line: Subjects with lower level of Telomerase.

will be increased in the cytoplasm if there is a cell damage. (40) Therefore, level of Cytochrome C should be further investigated at a correct time point after radiotherapy.

A scoring model to predict the radiotherapy response was calculated based on Z value of multivariate analysis. The coefficient of variable beta scoring that correlated (p -value<0.5) with the radiotherapy response was coefficient of Survivin: $(2.65/3.89) \times 10 = 6.8$ and coefficient of Telomerase: $(3.89/3.89) \times 10 = 10$. So the scoring model is $6.8S + 10T$ with S for Survivin and T for Telomerase. The scoring model cut-off point is ≥ 13.4 to determine the prediction of negative response. This cut-off point has values of LR+ 2.08, specificity 85.1%, sensitivity 31.0% and accuracy 58.5%. From the value of LR+ can be calculated the probability value after test is 67.5%. The probability of patients with a predictor score of ≥ 13.4 had a negative response increased to 67.5%. The predictor score of ≥ 13.4 is a sufficient diagnostic test to predict a negative response in stage IIIB cervical cancer patients.

Conclusion

Radiotherapy response is significantly correlated with Survivin and Telomerase levels of subjects with stage IIIB cervical squamous cell carcinoma. In addition, the subjects with lower Survivin or Telomerase level, have higher 1-year survival rate. Taken together, Survivin and Telomerase could be potential predictors of radiotherapeutic response for subjects with stage IIIB cervical squamous cell carcinoma.

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