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Correlation Between Akt and p53 Protein Expression and Chemoradiotherapy Response in Cervical Cancer Patients

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Akt is a protein that is associated with cell proliferation and is expressed at high levels in cancer cells. Some research indicates it may play a role in increasing the resistance of cancer cells to chemotherapy treatment. P53 is a tumor suppressor protein that influences the cell cycle and apoptosis. The purpose of this study was to examine the relationship between the expression of Akt and p53 in cancerous tissue before chemoradiation treatment, and the clinical response to treatment of cervical cancer patients. Twenty microscopic tissue samples were taken from cervical cancer biopsies obtained from patients before cancer treatment. The tissue samples were stained with p53 and Akt antibodies via immunohistochemistry technique, to measure expression of both proteins. After completion of chemoradiotherapy, patients' clinical response to treatment was determined using the pelvic control method. Our results revealed no correlation between expression of Akt and p53 index ($P = 0.74$) as well as between p53 Index and chemoradiotherapy clinical response ($P=0.29$). There was significant correlation between expression of Akt and cervical cancer chemoradiotherapy response ($P = 0.03$). There was no correlation found between p53 index and chemoradiotherapy clinical response ($P = 0.29$). High expression of Akt may related with high cell proliferation and resistance to chemoradiotherapy.

Key words: Akt, p53, cervical cancer, chemoradiotherapy

INTRODUCTION

Cervical cancer is the most common type of malignancy diagnosed in Indonesia. In general, patients seek treatment when the disease is not yet metastasized, but already at an advanced stage in its original location. In Indonesia, the treatment of choice for this type and stage of cancer is radiotherapy combined with chemotherapy or concurrent chemoradiotherapy (Didit & Rukmini 2002; Mufyala & Wofson 2008).

Radiotherapy provides significant curative and palliative benefits and is used in about 40-50% of all cases of cervical cancer (Delaney *et al.* 2005). The efficacy of radiotherapy is influenced by two factors i.e. physical and biological factors. Two important biological factors are the patient's intrinsic rate of cell proliferation, as well as the extent of hypoxia in

the cancer cells (Wilson *et al.* 2006). Physical factors include cell kinetics: the rate of proliferation in cancer cells correlates positively with its radiosensitivity and death by irradiation. The risk of cancer cell repopulation is greater after a failure of radiation treatment (Wilson 2003; Eriksen *et al.* 2004; Pedicini *et al.* 2012).

Akt [protein kinase B (PKB)] is a serine threonine protein kinase with oncogenic and anti apoptotic activities (Gasinska *et al.* 2004). This protein consists of Akt1, Akt2, and Akt3, all of which have been found to play a role in the regulation of fundamental cellular functions such as cell proliferation, survival and programmed cell death; glucose metabolism, ribosomal function, genetic transcription, and cell migration via phosphorylation of a multitude of substrates. Dysregulation of Akt signaling pathways featured in many human malignancies (Song *et al.* 2005; Ree 2008; Taylor *et al.* 2008). In cervical cancer, the increased expression of Akt results in the

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transformation of normal cervical to neoplasm cell (Crowell *et al.* 2007).

The p53 gene acts as a tumor suppressor. It works to regulate cell proliferation by stimulating transcription of other genes specific to cell cycle control (Brainwaite & Prives 2006) and the process of apoptosis (Bai & Zhou 2006). Cells with a wild-type p53 gene have normal cell cycle function, including the ability to shut down replication of abnormal DNA during the G1 to S phase. Cancer cells with an inactivated, or mutant p53 protein cannot function properly and so replication of abnormal DNA is not prevented. In fact, inactivation of wild-type p53 gene represents the most common genetic mutation implicated in human carcinogenesis (Fei & El Deiry 2003; Brenna *et al.* 2004), and some researchers maintain that its over-expression, as detected by immunohistochemistry, indicates a worse prognosis in some malignancies (Wootipoom *et al.* 2004; Kocak *et al.* 2013; Min *et al.* 2014).

Cancer cell death in radiotherapy is caused in part by apoptosis, a process which is also regulated by p53. A proposed association between Akt expression and the effectiveness of radiotherapy and chemoradiotherapy on cervical cancer is still controversial. Some studies report a positive correlation between Akt expression and prognosis/response, while others have found a negative correlation. In the majority of studies, the expression of Akt is observed qualitatively using immunohistochemistry method (Kim *et al.* 2006; Faried *et al.* 2006; Zhang & Zhang 2008; Kocak *et al.* 2013). The aim of the present research was to detect and evaluate any relationship between the expression of Akt in cervical cancer tissues measured by the IRS

(Immuno Reactive Score) method and p53 index with cervical cancer chemoradiotherapy response.

MATERIALS AND METHODS

Patients. Twenty consecutive subjects, were selected from among a whole series of 60 patients suffering from non-metastatic localized cervical carcinoma (stage IIB-IIIB). The subjects received a complete course of treatment, and were studied prospectively from July 2010 to March 2011 (Table 1). All patients were diagnosed and treated by definitive and concurrent chemoradiotherapy at Cipto Mangunkusumo Hospital (Jakarta) and Hasan Sadikin Hospital (Bandung) and provided written informed consent. Our research protocol was approved by the Research and Ethical Committee from Faculty of Medicine, University of Indonesia. Clinical staging of the patients was conducted by speculoscopy, bimanual examination and cystoscopy or rectoscopy, if necessary. Abdominal pelvic CT scans and chest X-rays were performed on all patients. Histological grade was classified according to the guidelines published by the Union for International Cancer Control as follows: G1 well differentiated; G2 moderately differentiated; and G3 poorly differentiated or undifferentiated. All patients had squamous cell carcinoma tumors with 13 patients diagnosed at clinical stage IIB, 1 patient at stage IIIA and 6 patients at stage IIIB.

Treatment. Patients were treated with a combination of External Beam Radiotherapy (EBRT) with ^{60}Co gamma rays and ^{192}Ir High Dose-Rate Intracavitary Brachytherapy (HDR-ICBT). EBRT

Table 1. Expression of Akt, p53 index, and chemoradiotherapy clinical response in cervical cancer treated with chemoradiotherapy

ID	Stage	IRS of Akt	p53 index	Response	Response group
A	IIB	12	0.21	bad	1
B	IIIB	4	0.36	bad	1
C	IIB	6	0.36	bad	1
D	IIIB	2	0.17	good	2
E	IIB	2	0.26	good	2
F	IIB	2	0.48	good	2
G	IIIA	4	0.67	good	2
H	IIIB	4	0.65	good	2
I	IIIB	3	0.38	good	2
J	IIIB	8	0.60	bad	1
K	IIB	3	0.36	bad	1
L	IIIB	6	0.59	good	2
M	IIB	6	0.28	bad	1
N	IIB	6	0.66	good	2
O	IIB	6	0.48	good	2
P	IIB	6	0.54	good	2
Q	IIB	4	0.44	good	2
R	IIB	4	0.43	good	2
S	IIB	4	0.39	good	2
T	IIB	4	0.13	good	2

was administered to the whole pelvis with a clinical target volume that included the primary cancer, uterus, internal iliac artery, pre sacral, external iliac artery, and lower common iliac lymph nodes. This was usually achieved by a “four-field box technique,” or sometimes by antero-posterior and postero-anterior parallel opposed portals. The usual field borders for anterior and posterior fields were superior at the L4-L5 inter space, inferior at the bottom of the obturator foramen or 2 cm distal from the tumor, and 1.5 to 2.0 cm lateral to the bony pelvic wall. Lateral fields had their anterior border at the symphysis pubis and the posterior border encompassed the entire sacral silhouette. A total dose of 50 Gy was prescribed in 25 equal fractions to the isocenter. HDR-ICBT using a Microselectron (Nucletron International, Amsterdam, Netherlands) followed by EBRT in two fractions (850 cGy/fraction) at point A. Cisplatin was administered at dose of 40 mg/m² on day 1, 8, 15, 22, and 29; 2 hours or less prior to EBRT treatment given concurrently on the same days (Pearcey *et al.* 2002; Elizabeth *et al.* 2006; Palmer *et al.* 2012).

Clinical Chemoradiotherapy Response.

Clinical response to radiation treatment was evaluated by a radiotherapist and classified according to the Hong Criteria (Gonzalez *et al.* 2002) as follows: (i) NRT (no gross residual tumor); complete or nearly complete regression of pelvic tumor; non specific fibrosis; or granulation over the cervix. These results are designated in our research as a positive (good) clinical response to treatment. (ii) GT response (gross residual tumor): gross tumor or palpable nodularity on cervix, and/or palpable in duration on the parametrium. These results are designated as a negative (bad) clinical response to treatment.

Immunohistochemistry. The p53 index and expression of Akt were analyzed by performing immunohistochemistry of pre-treatment biopsy samples. These samples of tumor tissue were embedded in paraffin, then incubated with Akt antibody (Abcam, USA) applied at a 1:100 dilution and with anti p53 monoclonal antibody (Leica,

Novocastra, ready to used-p53-D07), over night at 5 °C, in a moist chamber, followed by post primary, post protein, and Novolink HRP system (Novolink) and revealed with DAB (Novolink) and counterstained with Mayer Hematoxylin. The primary antibody was omitted in one of slide as a negative control. Staining for p53, observed in the nucleus, was scored as percent of stained cells (Kurnia *et al.* 2009). Up to 1000 cells were counted in each slide. Expression of Akt found in nucleus, cell cytoplasm and membrane, was observed in zones of maximum expression of the marker in at least 10 high power fields (400×). Expression of Akt was described using semi-quantitative scoring with the Immuno Reactive Score (IRS). The semi-quantitative analysis of the stained sections was carried out by light-microscopy and scored according to the IRS system introduced (Kaemerer *et al.* 2012) (Table 2).

Statistical Analysis. The p53 index was analyzed by using the Kolmogorov test for normal distribution data. Statistical analysis was conducted to assess any correlation between the Akt IRS score and the p53 index. Analysis of variance (ANOVA) test was used to analyze the relationship between the Akt IRS score and the clinical response of subjects to chemoradiotherapy. All statistical analyses were performed using Medcalc Software Version 9.2.0.1 (Harris & Taylor 2008).

RESULTS

In terms of clinical response to radiation treatment, 14 patients (70%) showed a positive clinical response and 6 patients (30%) showed a negative clinical response after completion of treatment (Table 2). Our goal was to determine whether there exists any correlation between these outcomes with Akt and p53 expression.

Measures of both Akt and p53 expression were obtained from tumor samples from 20 subjects. Expression of Akt was observed as brown coloration in the cancer cell cytoplasm (Figure 1A,B,C) and

Table 2. Akt and IRS classification scoring systems

Percentage of positive cells	Intensity of staining	IRS (0 – 12)
0 = no positive cells	0 = no colour reaction	0 – 1 = negative
1 ≤ 10% of positive cells	1 = mild reaction	2 – 3 = mild
2 = 10-50% positive cells	2 = moderate reaction	4 – 8 = moderate
3 = 51-80% positive cells	3 = intense reaction	9 – 12 = strongly positive
4 ≥ 80% positive cells		
IRS points		IRS classification
0 – 1		0 = negative
2 – 3		1 = positive, weak expression
4 – 8		2 = positive, mild expression
9 – 12		3 = positive, strong expression

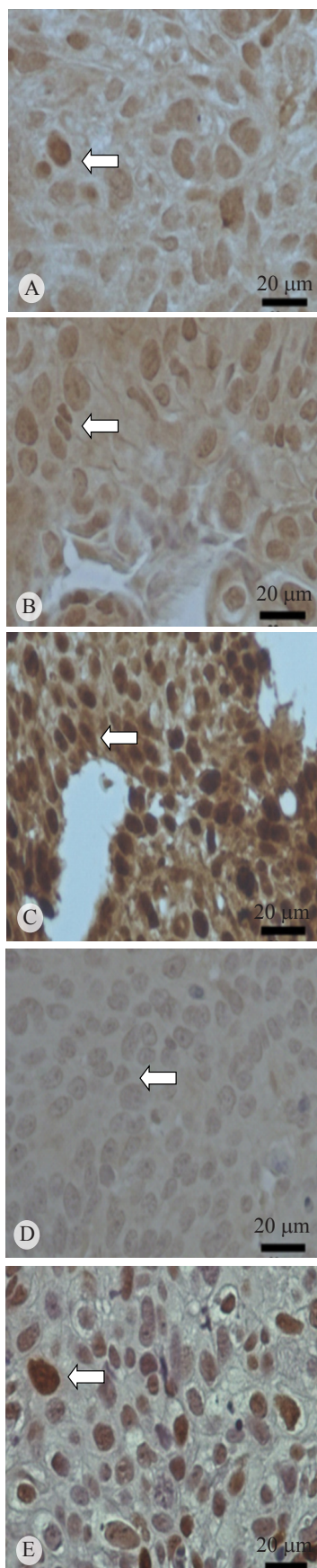


Figure 1. Expression of Akt is weak (A), medium (B) and strong (C) in cervical cancer tissues before treatment with chemoradiotherapy. Negative control (D) and expression of p53 (E).

Figure 1D as negative control. We observed strong expression of Akt in one patient (5%), mild-positive expression in 14 patients (70%), and low or negative expression of Akt in 5 patients (25%).

Patients who showed a positive clinical response to radiation treatment were #4 (28%) showed weak expression of Akt, and #7 (50%) showed mild expressions of Akt. Patients who had a negative clinical response to radiation treatment, #1 (14%) showed strong Akt expression, and #6 (85%) showed mild expression of Akt.

The expression of p53 protein can be observed as brown coloration in the nucleus (Figure 1E) and is measured as percentage of nucleus affected. Among our research subjects, the p53 index varied from 13 to 67% (mean 41%). As shown in Figure 2, we found no statistical correlation between expression of Akt and expression of p53 ($P = 0.73$, $r = 0.08$). We did find a significant correlation between higher expression of Akt and negative clinical response ($P = 0.03$) and between higher expression of p53 and positive clinical response ($P = 0.29$) after completion of chemoradiotherapy. Our results indicate that high expression of Akt before treatment correlates to negative clinical responses in chemoradiotherapy treatment.

DISCUSSION

Expression of Akt in our patient samples can also be categorized as either positive, or weak/negative expression, with a “positive” defined as $\geq 75\%$ of cervical cancer tissue testing positive for Akt expression; and “weak/negative” defined as $\leq 25\%$ of cancer tissue testing with low or no Akt expression. Other studies have analyzed pre-treatment tissues of patients with locally advanced cervical cancer and found that 86% (Kocak *et al.* 2013), and 74% (Kim *et al.* 2006), classify as “positive” for Akt. These studies used a different method to analyze the expression of Akt, which classified according to the intensity and quality of staining from Akt inside the cancer cell (Kim *et al.* 2006; Suzuki *et al.* 2010). In a study of glioblastoma (Suzuki *et al.* 2010) Akt was found in the nucleus as well in cytoplasm, with 29.7% of the subjects categorized as 50% high positive.

Our results showed only a weak, non-significant negative correlation between Akt and p53 expression. Prior research suggests that Akt will suppress expression of p53 related with mitochondria function. In wild-type p53, Akt clearly functions to modulate the direct action of p53 on the caspase-dependent mitochondrial function in death pathway (Yang *et al.* 2006). The relationship between Akt and p53 activity

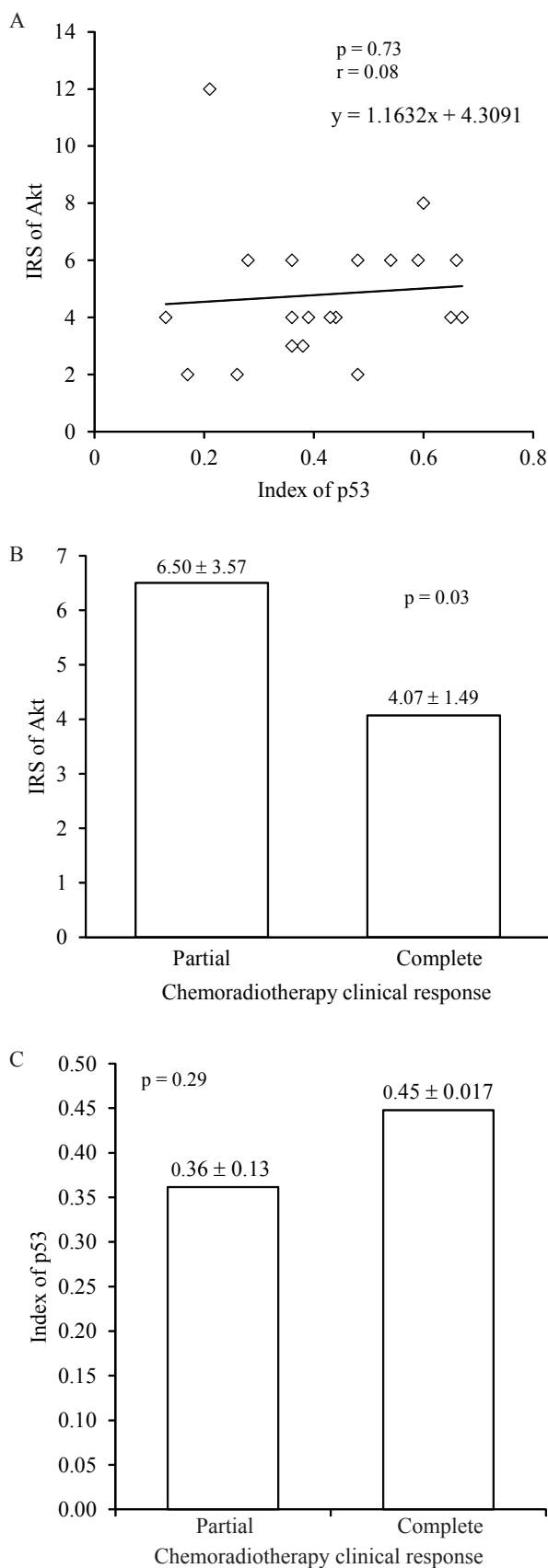


Figure 2. Correlation between expression of Akt, and p53 (A), expression of Akt (B), and p53 (C) in complete and partial response of chemoradiotherapy.

also influenced by MDM2 function by degradation of p53 protein (Zhou *et al.* 2001; Yoko *et al.* 2002; Milne *et al.* 2004). Although almost all p53 mutations can be detected using immunohistochemical techniques, sequencing analysis is still necessary to ensure the type of DNA mutation.

Expression of p53 and Akt each showed a different association with chemoradiotherapy clinical response. Higher expression of p53 tended to correlate with positive clinical response; it may be that mutated forms of p53 promote uncontrolled proliferation of cancer cells, which in turn increases radiosensitivity during the cell cycle phase, making the cells more susceptible to radiation than wild-type p53. Researchers have observed resistance to chemotherapy and radiotherapy in NSCLC (non small cell lung carcinoma) cell lines, when Akt is highly expressed (Brognard *et al.* 2001). Other studies have sought to understand and clarify the relationship between Akt and clinical response, in head and neck cancer treated with radiotherapy, and breast cancer treated with chemotherapy. Clinical studies have confirmed an association between Akt expression and resistance to radiotherapy in head and neck tumors (Gupta *et al.* 2002) and to chemotherapy in breast cancer (Stall *et al.* 2003). In this study, we surmise that Akt may disrupt the apoptosis process induced by irradiation that should lead to cancer cell death. One study of Non Small Cell Lung Carcinoma (NSCLCs) also showed a correlation between high expression of Akt and indicators of poor prognosis, in NSCLCs with lymph-node involvement, but it can't be considered to indicate distant of metastasis (Hirami *et al.* 2004).

Radiation exposure produces highly reactive free radicals, such as reactive oxygen species, that can interact with and damage DNA interfering with the cell's ability to reproduce normally. Reactive molecules may also affect intracellular functions such as cytokine production, growth factors, gene transcription, and apoptosis (Benz & Yau 2008; Flohe & Flohe 2011). Akt may contribute to treatment resistance by stimulating Non Homolog End Joining (NHEJ) repair and promoting cancer cell survival (Toulany *et al.* 2008) or by associating with DNA-PK in response to genotoxic stresses (Bozulic *et al.* 2008). Thus Akt can inhibit imminent cell death or apoptosis that would normally occur as a result of radiation treatment. PI3K/AKT signaling may influence clinical response to radiation in various ways, by altering regulation of mitochondrial proteins,

transcription factors, translation machinery, and cell-cycle progression (Zhan & Han 2004; Cheung & Testa 2013). Our research clearly supports the idea that the level of Akt expression prior to treatment can serve as a parameter to predict positive or negative clinical response to chemoradiotherapy in cervical cancer cases. High expression of Akt correlates to increased resistance of cervical cancer cells to chemoradiotherapy. Pre-treatment measurements of Akt expression can be used as a biomarker of patient response to chemoradiotherapy for cervical cancer.

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