RESEARCH ARTICLE

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High *ALDH-1* Expression Predicts Non-Complete Response of Radiotherapy in Stage III Squamous Cell Cervical Carcinoma Patients

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Abstract

Background: ALDH1 is a cervical cancer stem cell marker that has radioresistance profile. Recurrence and metastasis following radiotherapy are still being problems of most patients. This study aimed to determine the correlation between ALDH1 and radiotherapy response in stage III squamous cell cervical carcinoma (SCCC) of the cervix. **Methods:** A total 58 of 360 patients of stage III SCCC who received external beem radiation and brachytherapy (2016-2021) at Cipto Mangunkusumo Hospital met the eligibility criteria of this study. Pre- and post-irradiation MRI examinations and ALDH expression with immunohistochemistry (Santa Cruz®) were performed on formalin-fixed paraffin-embedded of pre-treatment cervical tissue biopsy taken from RSCM pathological anatomy laboratory. Patients were divided into two groups, complete responders vs non-complete responders. *ALDH-1* scores were compared between two groups to assess *ALDH-1* expression. The statistical analyses were carried out by SPSS 24. **Results:** The optimal *ALDH-1* score cut-off point on the radiation response was 166.05 pg/mL which was obtained from the analysis of the ROC curve. The AUC value was 0.682 with sensitivity and specificity, 63,6% and 64%, respectively. ALDH score ≥166.05 increased the risk by 3.127 times for not achieving complete response (adj OR 3.127, 95% CI 1.034 – 9.456, p = 0.043). Pre-radiation tumor size (p = 0.593), degree of differentiation (p = 0.161), renal abnormalities (p = 0.114), and keratinization (p = 0.477) were not associated with radiation response. **Conclusions:** High ALDH expression was associated with non-complete radiation response in stage III squamous cell cervical carcinoma.

Keywords: Squamous cell cervical carcinoma- complete response of radiation- ALDH-1- immunohistochemistry

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Introduction

Globally, cervical cancer is the fourth most common malignancy among women. In 2018, there were 570,000 new cases of cervical cancer and 311,000 deaths worldwide (WHO, 2014). From the American Cancer Society, there were 14,480 new cases of cervical cancer and 4,290 deaths in the United States in 2020. Meanwhile, In Indonesia, there were 36,633 new cases with 21,003 deaths among women with cervical cancer (Sung et al., 2021).

The diagnosis of cervical cancer in Indonesia, frequently delayed. Most patients come to the hospital at advanced stage. The study of Nuranna and Fahruddin (2019), in Dr. Cipto Mangunkusumo General Hospital, 1,303 cervical cancer patients 10.5%, 25.7%, 54.3% and 9.5% came at stage I, II, III and IV (Nuranna and Fahrudin, 2019). Radiotherapy is one of the treatments for locally advanced stage cervical cancer. Recurrence and metastasis after radiotherapy are still being problems of most patients. Some tumor markers were postulated being prognostic factors in cervical cancer, including serum-associated antigen, angiogenesis, apoptosis, DNA repair, hypoxia, proliferation, metabolism, and stem cell marker (Yao et al., 2020).

Recently, there have been several studies showing that cancer stem cells are radioresistant in cervical cancer (Yao et al., 2020). The response of radiation between cancer stem cells and non-cancer stem cell is different. The volume of

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non-cancer stem cell is reduced due to DNA damage after radiation. Self-repair capabilities via ATM and Chk1/2 are more efficient in stem cells. Rapid repopulation during and after radiation is an important factor in radiation failure. Stem cells show increased activity of ROS scavengers, thereby lowering Reactive Oxygen Species (ROS) (Rachmadi et al., 2019; Cojoc et al., 2015). Aldehyde dehydrogenase 1 (*ALDH1*) is a cervical cancer stem cell marker that shows radioresistance characteristics. *ALDH1* is a detoxification enzyme involved in aldehyde metabolism, both endogenous and exogenous, which can reduce oxidative stress in prokaryotic and eukaryotic organisms (Huang and Rofstad, 2017).

Based on the references, study on the relationship between *ALDH1* and radiation response in cervical squamous cell carcinoma has never existed. In this study,

ALDH is expected to act as a marker to predict radiation response in cervical squamous cell carcinoma. If it is proven, further research can be carried out for the development *ALDH-1* of targeted therapy to overcome radioresistance (Chen et al., 2009).

Materials and Methods

This study was conducted by collecting data from the cancer registry at the Anatomical Pathology Department of Dr. Cipto Mangunkusumo Hospital (RSCM) with the keywords "squamous cell carcinoma of cervix stage III" from 2016 until 2021. The inclusion criteria were squamous cell cervical carcinoma stage III patient who had good quality of formalin-fixed paraffin-embedded of pre-treatment cervical tissue biopsy taken in RSCM, completed radiotherapy, and had a report of radiotherapy response. We excluded the women who had undergone other treatments (such as surgery or chemotherapy), and incomplete data. This study is already being allowed by the Ethics Committee [Reference number: KET- 378/UN2. F1/ETIK/PPM.00.02/2021] and fulfilled the Declaration of Helsinki.

Based on cancer registry data, 360 subjects were obtained. A total 277 of 360 subjects did not meet the inclusion criteria due to incomplete data: no pre-radiation or post-radiation MRI evaluation, did not have complete radiation, the patient had a history of radiation or previous chemotherapy, and the patient had a history of other malignancies. Furthermore, 23 of 83 subjects could not be included in this study because they did not have paraffin blocks or Haematoxillin-eosin (HE) slides. There were 60 subjects who met the inclusion and exclusion criteria. After being reassessed by an expert anatomical pathologist, there were 2 subjects who could not be included because of the histopathological type of adenocarcinoma and not from the cervix. The final number of subjects included in this study were 58 samples.

Radiotherapy response was evaluated based on the Response Evaluation Criteria in Solid Tumors (RECIST) in 3 months after completing radiotherapy by magnetic resonance imaging (MRI) examination. Complete response was defined if no measurable primary tumor lesion, no new tumor growth, or no associated symptoms. Meanwhile, non-complete responses were divided to partial response (a <30% decrease in the tumor size, no new lesion, no disease progressivity), progressive response (a >20% increase in the tumor size), and stable response (neither included in partial nor progressive response). ALDH expressions were assessed using immunohistochemistry (Santa Cruz®). This examination use immunoperoxidase methods on unstained slides of formalin-fixed paraffin-embedded (FFPE). The 3 – 5 μm section block of tissue biopsy was used as the positive control, then followed the manufacturer's guidance. The primary antibody was *ALDH1*A1 ([5A11] ab105920, Abcam). The negative control made by skipping the primary antibody administration step. The evaluation of immunostaining carried out using 100 and 400 times magnifications by light microscopic. H-score was assessed by combining the cytoplasmic staining intensity (i, 0 = no evidence of staining, 1 = weak staining, 2 = medium staining, 3 = strong staining) and the percentage of stained cells at each intensity level (Pi, varies from 0% to 100%). The final H-score was obtained from the equation (0 x P0) + (1 x P1) + (2 x P2) + (3 x P3) with result range 0-300 (Figure 1). The reliability test was done for validation of immunohistochemistry (IHC) result. The reliability test was 0.968 (p 0.001).

Statistical analysis

The patient characteristics were summarized using descriptive statistics. Continuous variable with normal distribution presented as means with standard deviation (SD). The data with no normal distribution served as median with the minimal and maximal scores. Meanwhile, the categorical variables served as proportions. The analyses were performed using SPSS 24 software. Univariate and multivariate analyses evaluate the associations between clinicopathological factors and radiotherapy response. The area under the curve (AUC) used to analyze the receiver operating characteristic (ROC) curve.

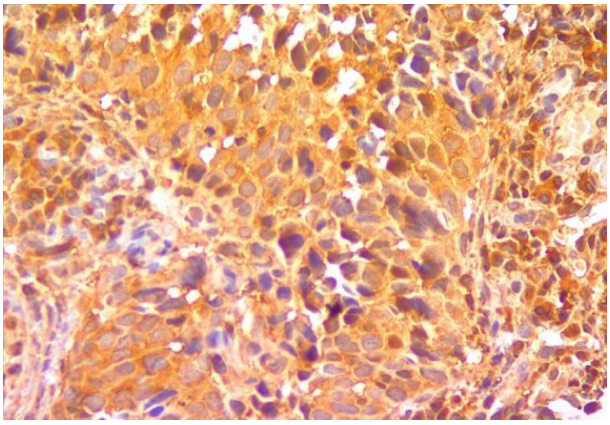
Results

A total 58 of 360 eligible subjects who met the criteria were further analyzed. The study subject characteristics shown in Table 1. The mean ALDH level was 165.745 pg/mL (SD 23.322). A 56.9% of subjects had no complete response. Meanwhile, 43.1% subjects have a complete response.

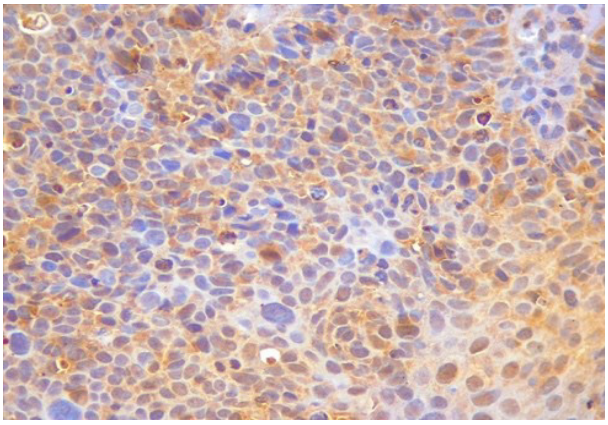
The determination of the cut-off value was carried out to assess the ability of the ALDH score to distinguish complete and incomplete responses using the Receiver Operating Characteristic (ROC) curve with result 0.682. The optimal sensitivity and specificity of ALDH scores on radiotherapy response are 0.636 and 0.640 with optimal cut-off value is 166.05 (Figure 2).

Correlation of clinicopathological factors and radiotherapy response

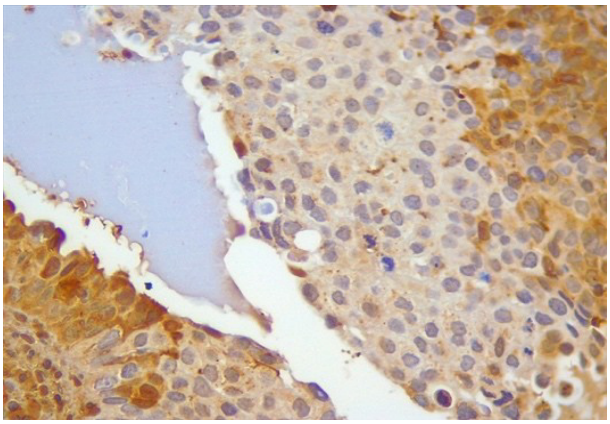
A total of 63.6% of patients with non-complete response had an ALDH score ≥ 166.05, meanwhile in complete response group was 36%. The results showed a statistically significant relationship between the ALDH score and the radiation.



High ALDH score (197.20)



Moderate ALDH score (157.44)



Low ALDH score (121)

Figure 1. ALDH Expression Using H-score of Immunohistochemistry

Response (p value <0.05). ALDH score 166.05 increased the risk up to 3 times for not achieving a complete response (OR = 3.111, 95% CI 1.055 - 9.176, p = 0.037). There was no correlation of pre-radiation tumor size (p 0.593), differentiation (p 0.161), renal impairment (p 0.114), and keratinization (p 0.477) with complete response of radiotherapy (Table 2).

Table 1. Characteristics of Study Subject

Variable	Value (n(%))
Age*	52.72±9.09
Parity$	3 (0-6)
Pre-radiation tumor size (mm)$	63.0 (25.0-150.0)
ALDH (pg/mL)*	165.745±23.322
Keratinization	
Yes	18 (31)
No	40 (69)
Grading	
Well differentiated	31 (53.4)
Moderate - poor differentiated	27 (46.6)
Renal impariment	
Yes	18 (31)
No	40 (69)
Radiotherapy response	
Complete response	25 (43.1)
No complete response	33 (56.9)

*mean (standard deviation/SD); $median (min – max)

Predictors of non-complete radiation response in stage III SCC cervical cancer patients were determined using the backward stepwise method of multivariate logistic regression. The variables included in the multivariate analysis were variables that had a p value of <0.25 in univariate analysis. In this study, those who had p < 0.25 were ALDH scores, kidney abnormalities and degrees of differentiation.

ALDH score had a significant relationship with radiation response (adj OR 3.127, 95% CI 1.034 – 9.456, p = 0.043). ALDH score might be predicted having a significant and independent relationship with radiation response. Patients with an ALDH score ≥ 166.05 increased the risk of not achieving a complete response up to 3 times higher than subjects with an ALDH score < 166.05 (Table 3).

Discussion

We found significant association between *ALDH1* and radiation response in which a high ALDH score has a 3 times risk of not achieving a complete radiation response. This study is the first in assessing association ALDH and radiation response in stage III Squamous Cell Cervical Carcinoma (SCC). The results are consistent with Javed et al., (2012) that stated ALDH expression in cervical

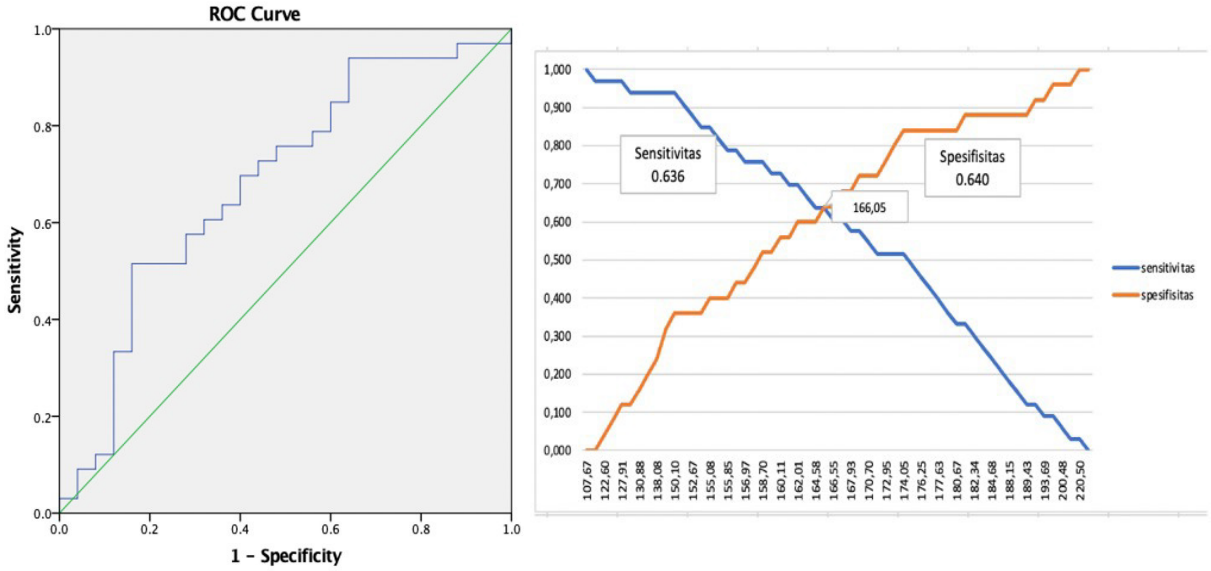


Figure 2. ROC Curve and Cut off Value of Sensitivity and Specificity of ALDH Score

Table 2. Correlation of Clinicopathological Factors with Radiotherapy Response

Variables	Response				OR	95% CI	P
	Complete		No Complete				
	n	%	n	%			
ALDH							
≥ 166.05	9	36.0	21	63.6	3,111	1.055-9.176	0.037
< 166.05	16	64.0	12	36.2			
Pre-radiation tumor size (mm)*	65.0 (37.0 - 130.0)		63.0 (25.0 - 150.0)				0.593
Differentiation							
Good	16	64.0	15	45.5	2,133	0.735 - 6.915	0.161
Moderate - poor	9	36	18	54.5			
Renal impairment							
Yes	5	20.0	13	39.4	0.385	0.1115 - 1.281	0.114
No	20	80.0	20	60.6			
Keratinization							
Yes	9	36.0	9	27.3	1,500	0.490 - 4.595	0.477
No	16	64.0	24	72.7			

*mean (standard deviation/SD); OR, odds ratio; CI, confidence interval

biopsy tissue before therapy was useful in predicting poor chemoradiation response. However, that study had different subjects, involving patients with histological types of SCC and adenocarcinoma (Javed et al., 2012). In contrast to the study of Javed et al., (2012) which did not mention the cut off ALDH score, our study found the cut off optimal ALDH score ≥ 166.05 had a risk to not achieving a complete therapeutic response.

ALDH1 is an enzyme found in the cytoplasm. ALDH is generally a marker of cancer stem cell (CSC) in cervical cancer and plays a role in poor prognosis. Overexpression of ALDH plays a role in cancer progression, which increases the potential for self-renewal and differentiation. High ALDH causes resistance to cervical cancer therapy (Jiang et al., 2008; Bertrand et al., 2014; Mihatsch et al., 2011). Radioresistance mechanisms are also possible

Table 3. Multivariate Analysis of Predictors of Radiotherapy Response in Squamous Cervical Cancer Stage IIIB

Variable	B	S.E.	Wald	df	Adj. OR	CI 95%	Sig
ALDH score ≥ 166,05	1.140	0.565	4.079	1	3.127	1.034 - 9.456	0.043
Pre-radiation renal impairment	(-)0.964	0.637	2.228	1	0.382	0.110 - 1.330	0.130
Konstanta	1.356	1.154	1.380	1	3.879		0.240

*B, (base); S.E, (standard error); Wald, (uji wald); df, (degree of freedom); Adj.OR, (adjusted odd ratio); CI, (confidence interval); Sig, (significancy)

due to the induction of hypoxic conditions. A hypoxic environment can directly protect CSC by depleting oxygen and support CSC survival and expansion through activation of the hypoxia-inducible factor (HIF) pathway. In prostate cancer studies, the expression of *ALDH1A1* and HIF is aligned (concurrently). This indicates that cells with high *ALDH1A1* can show radioresistance because they are protected by hypoxic conditions (Cojoc et al., 2015).

Radiation produces an increasing ROS. The production of ROS after radiation can cause cell death through damage to macromolecules such as DNA, proteins and lipids. However, in cells expressing high ALDH, this is not the case. Radiation- induced free radicals can be directly prevented by *ALDH1* by producing NAD(P)H (Cojoc et al., 2015; Singh et al., 2013).

Association between pre-radiation tumor size and radiation response is still controversial. In line with previous study by Siregar et al., and Kusuma et al., our study also showed that pre-radiation tumor size did not correlate with radiation response (Siregar et al., 2017; Kusuma et al., 2020). Another study by Rahakbauw and Winarto (2018) 76.92% cervical cancer patients stage IIA-IIIB with large tumor size (≥4cm) were 1.3 times having poor radiotherapy response. Study from Yang et al., (2019) stated that large tumor size is associated with rapid proliferation. Proliferating cells are target of radiotherapy. However, the statements still lack evidence.

In this study, most patients (64%) with well differentiation had a complete radiation response but were not associated significantly. Reagan and Fu (1979) stated that SCC of cervix patients with well differentiation have 21.3% of 5-years overall survival. The previous studies also stated no correlation between grading and complete radiation response (Rahakbauw and Winarto, 2018; Shah et al., 2018).

Our study found pre-radiation renal impairment did not associate with radiotherapy response. Meanwhile, study from Nuranna et al., showed most cervical cancer patients with renal impairment have higher progressive response (Nuranna et al., 2019). Rose et al., (2010) also stated from 539 of stage III cervical cancer patients with hydronephrosis had poor survival rates. Hydronephrosis often occurs in cervical cancer patients who have renal impairment. Obstruction in the urinary tract can affect the patient's prognosis. Thus, hydronephrosis is an independent predictor of cervical cancer survival. Cervical cancer patients with kidney disorders may not receive nephrotoxic agents (Cetina et al., 2004). Cervical cancer therapy may worsen kidney disease (Pradhan et al., 2011).

We found different results with previous study on correlation between several clinicopathological factors and radiotherapy response. It might be due to heterogeneous subject conditions, comorbidities, social factors, and varied nutritional state that we did not assess. In addition, the intrinsic radiosensitivity of tumor cell makes the different molecular characteristics. There were some limitations in our study. This study was conducted retrospectively in a single study center. The number of samples was relatively small because of incomplete data, unavailability of paraffin block, unavailability of pre- nor post- radiation MRI examinations and some patients did not complete radiation. The most common monitoring modality is ultrasound. Paraffin blocks were not available in 30-40% of subjects, because the biopsy was not performed at our hospital. So, the histopathological data used the review slides from the previous hospital.

In this study, high ALDH may increase the risk of not achieving a complete radiation response. Thus, there is an ongoing need to develop novel and specific ALDH inhibitors for effective cancer treatment. The author recommends conducting further research on ALDH expression in local tumor areas by comparing pre and post-radiation values with a larger number of samples and using prospective methods, conducting further studies with manipulation of ALDH expression using specific ALDH inhibitors in local tumor areas aimed to solve radioresistance problems.

In conclusions, High ALDH expression was associated with non-complete radiation response in stage III Squamous Cell Cervical Carcinoma. Patients with high ALDH levels increased the risk by 3 times for not achieving the complete response.

Author Contribution Statement

MNF and FK contributed for study ideas. MNF, FK, TH, AK, SMS, LN, GP, and TDA were designed the study. MNF, FK, and TH performed research course. MNF, FK, and AK performed data analysis and interpretation. MNF and FK drafted initial manuscript. All authors finalized the manuscript..

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Ethical Approval

This study is a part of an approved student thesis. All the data in this study was permitted by the Ethics Committee of Dr. Cipto Mangunkusumo General Hospital [Reference number: KET- 378/UN2.F1/ETIK/PPM.00.02/2021].

Availibility of Data

The data in this study could be accessed under permission of authors

Conflict of Interest

There was no conflict of interest declared by the authors.

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