

## RESEARCH COMMUNICATION

# p63 Cytoplasmic Aberrance is Associated with High Prostate Cancer Stem Cell Expression

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### Abstract

**Introduction:** Prostate cancer in Indonesia is the 3<sup>rd</sup> ranking cancer among males and the 5<sup>th</sup> rank for their cancer mortality. Prognostic markers that can identify aggressive prostate cancer in early stages and help select appropriate therapy to finally reduce the mortality are therefore urgently needed. It has been suggested that stem cells in the prostate gland have a role in initiation, progression, and metastasis of cancer, although controversy continues to exist. Maintenance of normal stem cell or reserve cell populations in several epithelia including prostate has been shown to be regulated by p63 and alteration of p63 expression is considered to have an oncogenic role in prostate cancer. We hypothesize that the expression of cytoplasmic aberrance of p63 is associated with high ALDH1A1 expression as a cancer stem cell marker, thus leading to progression of prostate cancer. **Methods:** Using a cross-sectional study during two years (2009-2010), a total of 79 paraffin embedded tissues of benign prostatic hyperplasia, PIN prostatic intraepithelial neoplasia, low and high Gleason score prostate cancer were investigated using immunohistochemistry. Associations between cytoplasmic p63 and ALDH1A1, as well as with pathological diagnosis, were analyzed by Chi-Square test using SPSS 15.0. Links of both markers with cell proliferation rate (KI-67) and apoptotic rate (cleaved caspase 3) were also analyzed by Kruskal-Wallis test. **Results:** The mean age of patient at the diagnosis is 70.0 years. Cytoplasmic aberrance of p63 was associated with ALDH1A1 expression ( $p < 0.001$ ) and both were found to have significant relationships with pathological diagnosis (including Gleason score), ( $p = 0.006$  and  $p < 0.001$  respectively). Moreover, it was also found that higher levels of cytoplasmic p63 were significantly associated with the frequency of proliferating cells and cells undergoing apoptosis in prostate cancers ( $p = 0.001$  and  $p = 0.016$  respectively). **Conclusion:** p63 cytoplasmic aberrance is associated with high ALDH1A1 expression. These components are suggested to have an important role in prostate cancer progression and may be used as molecular markers.

**Keywords:** p63 - ALDH1A1 - cancer stem cell - prostate cancer

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### Introduction

Prostate cancer is the second most frequently diagnosed cancer of men (899,000 new cases, 13.6% of the total) and the fifth most common cancer overall. Prostate cancer in Indonesia is the 3<sup>rd</sup> rank cancer in male (9033 new cases) and 5<sup>th</sup> rank in causes of cancer mortality in male (6841 cases) (Ferlay et al., 2010). The number of cases has continuously increased over the past decades (Eble et al., 2004). The treatment of prostate cancer depends on the stage of the disease, resulting in variable clinical outcome (Cookson et al., 2007). Prognostic markers that can identify aggressive prostate cancer in early lesion and help select appropriate therapy to finally reduce the mortality are therefore urgently needed. However, current techniques are not accurate enough to predict the outcome of prostate cancer. New diagnostic modalities have to be

developed (Cookson et al., 2007).

The cancer stem cell (CSC) hypothesis states that tumorigenesis is driven by CSCs that might be derived from mutated adult stem cells and driven by components that display stem cell properties (Pardal et al., 2003). Recent studies pertaining prostate cancer stem cells reveal that prostate CSCs representing a small percentage of the total tumor mass are much more tumorigenic than their progeny cells. They also have self-renewal capacity, more metastatic ability, and recapitulating the heterogeneity of the original primary tumor (Collins et al., 2005; Patrawala et al., 2006; Li et al., 2009; Van den Hoogen et al., 2010). Identification of CSC marker in prostate cancer is being developed. The new candidate of prostate cancer stem cells is being proposed. ALDH1, which may have a role in early differentiation of stem cells through its role in oxidizing retinol to retinoic acid, has been proposed for the perfect

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candidate of cancer stem cells in prostate (Magni et al., 1996; ; Yoshida et al., 1998; Duester, 2000; Sophos & Vasilidou, 2003; Chute et al., 2006). It has been proven that the ALDH1(+) prostate cancer cells have high clonogenic and tumorigenic capacities in *in vitro* and *in vivo* study. Furthermore in clinical study, the high ALDH1 expression in prostate cancer was positively correlated with Gleason score and pathologic stage, and inversely associated with overall survival and cancer-specific survival of the patients (Li et al., 2010).

How these prostate cancer stem cells are being initiated? This important question is being investigated by many researchers. It has been proposed by Reiner et al. the cells of origin for the initiation of prostate cancer are a subset of p63(+) basal epithelial cells (Reiner et al., 2007). As a homologue of p53, p63 has been proven to be correlated with the expression of one of stem cell markers, namely CD44, in some tissues. It has been demonstrated that p63 have a role in regulation of CD44 expression in breast epithelial cell line (Carroll et al., 2006). Regulation of CD44 by p63 has been also proven in squamous cell carcinoma of head and neck (Boldrup et al., 2007). The role of p63 in regulation of prostate cancer stem cells is not well defined. But, the alteration of p63 expression in prostate cancer has been proven to be correlated with cell kinetics (cell proliferation and apoptosis) and also prostate cancer-specific mortality (Dhillon et al., 2009).

Prostate cancer showed a unique precancerous lesion which is named Prostatic Intraepithelial Lesion (PIN). PIN can be detected microscopically in young males, its prevalence increases with age and PIN shows strong association with cancer in terms of coincidence in the same gland and in its spatial distribution (Eble et al., 2004). This transitional lesion from benign condition into malignancy might be used in disease modelling of cancer development.

In present study, the expression of cytoplasmic p63 and ALDH1 between epithelium of PIN and prostate cancer in different histological grade will be compared in order to give us insight into prostate cancer initiation and progression by a subset of cancer stem cells. Their association with cell kinetics (cell proliferation and apoptosis) will be also investigated. More importantly, these specific markers could be developed as a new diagnostic system for early detection and prognostic determination in progression of prostate cancer, and hence probably offering a target therapy to cure the challenging malignancy.

## Materials and Methods

### Subjects

The design of this research was a semi-quantitative non-experimental, performed by cross sectional method. The duration of study is 2 years (2009-2010). There was no follow up or reverse back intervention done in this research. Subjects used in this study were paraffin-embedded tissues of prostate cancer, prostatic intraepithelial neoplasia, and normal prostate (benign prostatic hyperplasia), which had been taken from Dr. Sardjito General Hospital (Pathology Laboratory archive). Paraffin-embedded tissues taken from prostate by core biopsy method or containing very

small focus of tumor (<5 fields of view) are excluded from the study. Hematoxylin Eosin slides were examined by two independent pathologists in order to classify normal prostate, PIN and prostate cancer. Grading was classified by using Gleason Grade of prostate cancer. Patients with Total Gleason Score  $\leq 7$  were considered as low Gleason grade. Meanwhile, patients with Total Gleason Score 8-10 were considered as high Gleason grade (Li et al., 2010)

### Immunohistochemical Staining

Those paraffin-embedded tissues were processed with immunohistochemical staining (IHC) following manufacturer's procedure. Antibodies used in this study were monoclonal antibody anti p63 (BC4A4, Biocare, dilution 1:100), ALDH1A1 (EP1933Y, Biocare, dilution 1:200), Ki-67(MM1, Biocare, dilution 1:100), and cleaved caspase 3 (Biocare, dilution 1:100). Positive control was taken from paraffin-embedded breast cancer tissue with positive IHC for ALDH1A1, normal prostatic gland of benign prostatic hyperplasia tissue for p63, and tonsil tissue for Ki-67. Paraffin-embedded tissue of prostate cancer without antibody was used as the negative control.

*Immunohistochemical results were analyzed based on following criteria.*

p63 aberrant cytoplasmic expression. The percent of positively stained for p63 is scored on a scale of 0% to 100% in 5 field of views, and are grouped in 3 tertiles (Dhillon et al., 2009).

ALDH1A1: Immunoreactive staining intensity of cells with ALDH1A1 positive staining in 5 fields of view are rated according to the following scale: no visible staining: 0, faint staining: 1, moderate staining: 2, and strong staining: 3. The percentage of cells with positive staining was graded as 0, <10, 10-25, 25-50, 50-75%, and >75%. Final score was obtained by multiplying both scores. ALDH1A1 expression was then stratified at three levels; absent (a specimen without any expression of ALDH1A1), present (a specimen that had <10% of cells for ALDH1A1 expression with faint staining), and a high level ) a specimen with more than 10% overall score) of ALDH1A1 expression (Ginestier et al., 2007, Li et al., 2010).

Ki-67 (MIB1): Ki-67 was used to identify proliferating cells. The Ki-67 score was assessed as the number of stained nuclei over the total number of tumor nuclei in 5 fields of view (Dhillon et al., 2009).

Cleaved caspase 3: Cleaved caspase 3 was used to identify the percent of tumor cells undergoing apoptosis. Apoptosis score was assessed as the number of positive cells out of total number of tumor cells in 5 fields of view (Vigneswaran et al., 2007).

### Statistical Analysis

The statistical software SPSS 15.0 was used to compare ALDH1A1 expression in some groups with particular characteristic. Crosstabulation were applied to generate descriptive tables. Differences of ALDH1A1 expression in ages, pathological diagnosis, and aberrant cytoplasmic expression of p63 were examined using Chi-square test ( $p < 0.05$ ). The percent of cells positive

for Ki-67 and cleaved caspase 3 were log transformed and analyzed across groups of high ALDH1A1 by Mann-Whitney test ( $p < 0.05$ ). Meanwhile, Ki-67 and cleaved caspase 3 expression were analyzed across tertiles of p63-positive area by Kruskal-Wallis test ( $p < 0.05$ ). This study was approved by the Institutional Review Board at Faculty of Medicine, Gadjah Mada University, Indonesia.

## Results

### Characteristics of samples

In this study, 79 prostate specimens were collected. Patients had mean age at the diagnosis of 69.89 years, with minimum age 50 years and maximum 85 years. Among the population, prostate cancer patients had mean age 70.93, with minimum and maximum age 50 and 85 years consecutively. Majority of tissue samples were collected from transurethral resection of prostate ( $n = 76$ ). The rest of samples were collected from open prostatectomy ( $n = 3$ ). From 79 samples collected, 16 samples are belonged to Benign Prostatic Hyperplasia (BPH), 18 samples prostatic intraepithelial neoplasia (PIN), 20 samples prostate cancer with Gleason score  $\leq 7$ , and 25 samples prostate cancer with Gleason score 8-10.

### ALDH1A1 Expression

All BPH tissues as representative of normal prostate expressed  $\leq 10\%$  population of ALDH1A1 positive cells with faint staining (Table 1). Thus,  $>10\%$  overall score could be used as a cut-off. In other words, a tumor specimen with more than 10% overall score was defined as one with high ALDH1A1 expression. High ALDH1A1

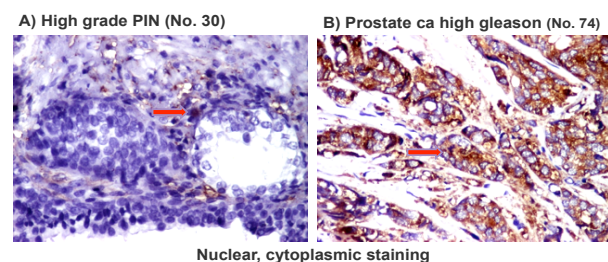
expression was found in 38% of total samples ( $n=79$ ). Two out of 18 (11.1%) samples of PIN showed high ALDH1A1 expression (Figure 1A). Among prostate cancer specimens ( $n=45$ ), high ALDH1A1 was expressed in 62% population (Figure 1B). Thus, we found different expression of high ALDH1A1 between BPH, PIN, and prostate cancer with different Gleason score ( $p<0.001$ ). This difference were also found in age of patients ( $p=0.025$ ). Among prostate cancer specimens, however, ALDH1A1 expression were not significantly associated with cancer cell proliferation and apoptosis (Figure 1).

### Aberrant Cytoplasmic of p63 Expression

In this study cytoplasmic staining of p63 in BPH, PIN lesion, prostate cancer cells were observed (Table 2). This is a rare expression pattern for a protein that is normally absent in prostate adenocarcinoma and that is usually strongly expressed in basal cells of benign prostate glands (Figure 2A). The mean of the area displayed p63 immunoreactivity is 11.3% with maximum score of 77.6%. Predominantly samples of BPH and PIN had expressed no cytoplasmic p63 (only one sample of BPH expressed this protein in its cytoplasm). Among prostate cancer (Figure 2B), significant association between p63 cytoplasmic staining tertiles and Gleason score was found ( $p=0.006$ ). In the same histologic specimens (Figure 3), higher levels of cytoplasmic p63 were significantly associated with higher frequency of proliferating cells ( $p=0.001$ ) and, interestingly, were also associated with higher proportion of cells undergoing apoptosis ( $p=0.016$ ). The association between cytoplasmic p63 tertiles and age of patients meet no significant result.

**Table 1. Immunohistochemical Staining of ALDH1A1 on 79 Prostate Specimens with Clinicopathological Characteristics and Cytoplasmic p63**

Characteristics	No. of samples	High ALDH1A1	P value
All cases	79	30	
Age (years)			0.025
$\leq 65$	28	6 (21.4%)	
$> 65$	51	24 (47.1%)	
Pathological Diagnosis			0.000
BPH	16	0	
PIN	18	2 (11.1%)	
Prostate ca. (Gleason $\leq 7$ )	20	10 (50%)	
Prostate ca. (Gleason 8-10)	25	18 (72%)	
Aberrant cytoplasmic p63			0.000
Absent	44	6 (13.6%)	
Low	17	10 (58.8%)	
High	18	14 (77.8%)	

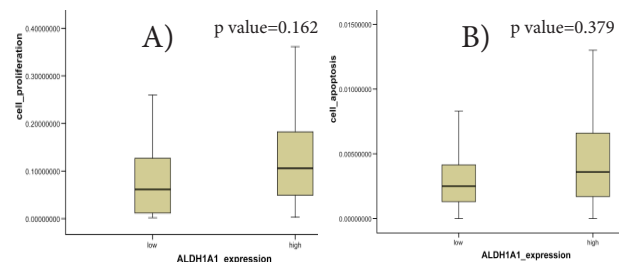


**Figure 1. A) ALDH1A1 was in Few Cells of PIN, B) Several Number Prostate Cancer Cells.**

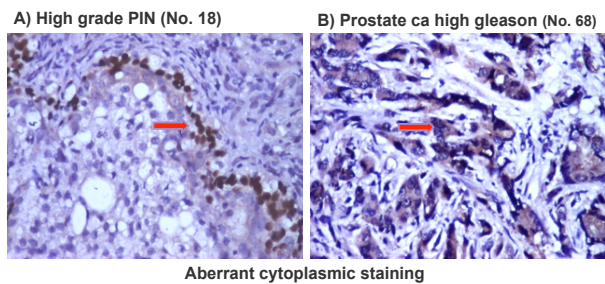
**Table 2. Immunohistochemical Staining of Cytoplasmic p63 on 79 Prostate Specimens with Clinicopathological Characteristics.**

Characteristics	No. of samples	Cytoplasmic p63		
		Absent	Low	High
All cases	79	44	17	18
Age (years)				(Pvalue=0.140)
$\leq 65$	28	19 (6.79%)	6 (21.4%)	3 (10.7%)
$> 65$	51	25 (49%)	11 (21.6%)	15 (29.4%)
Diagnosis				(Pvalue=0.000)
BPH	16	15 (93.8%)	1 (6.3%)	0
PIN	18	18 (100%)	0	0
Prostate ca.*	20	8 (40%)	9 (45%)	3 (15%)
Prostate ca.**	25	3 (12%)	7 (28%)	15 (60%)

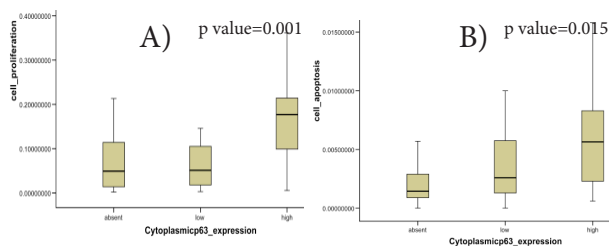
\*Gleason  $\leq 7$ , \*\*Gleason 8-10



**Figure 2. Association between ALDH1A1 Expression and Cancer in 45 Prostate Cancer Specimens. A) Cell Proliferation, B) Cell Apoptosis.**



**Figure 3. A) Nuclear p63 was Expressed in the Basal Cells of PIN, B) Meanwhile, Cytoplasmic p63 was Expressed in Prostate Cancer Cells.**



**Figure 4. Association between Cytoplasmic p63 Expression and Cancer in 45 Prostate Cancer Specimens. A) Cell Proliferation, B) Cell Apoptosis.**

*Association between ALDH1A1 and Cytoplasmic p63*

Both high ALDH1A1 and cytoplasmic p63 were not expressed in BPH and PIN specimens. In this study, high ALDH1A1 expression was found to be significantly associated higher level of cytoplasmic p63 tertiles ( $p < 0.001$ )

**Discussion**

In this study, all BPH specimens had <10% of cells expressing ALDH1A1. This finding is consistent with previous study conducted by Li et al. Therefore,  $\geq 10\%$  overall score to define high ALDH1A1 expression could be also used as a cut-off in this study (Li et al., 2010). The number of prostate cancer specimens expressing ALDH1A1 (38%) is higher than in previous study, which is only 20% of population (Li et al., 2010). High frequency of ALDH1A1 expression had also been found in ovarian cancer, in which 28.9% tumors had 21-100% staining and 44% tumors had 1-20% staining (Landen et al., 2010).

We found a significant association between high ALDH1A1 expression with pathological diagnosis (including Gleason score) and age of patients. Previous study had proved the association between ALDH1A1 expression with histological grade but not age of patients (Ginestier et al., 2008, Li et al., 2010). We failed to relate ALDH1A1 expression with cancer cell proliferation and apoptosis. However the association of ALDH1A1 expression with histological grade still important to be outlined since it has a great impact to several histopathological and clinical outcomes (reviewed in Humphrey, 2004). The presence of high ALDH1A1 positive cells in benign lesion need a special attention since it may have high capability of self renewal as been proven in breast epithelium (Ginestier et al., 2008).

The cancer stem cell (CSC) hypothesis states that CSCs might be driven by components that regulate normal

stem cells (Pardal et al., 2003). In this research, we tried to figure out the relationship between the expressions of ALDH1A1, as cancer stem cell marker, and ectopic expression of p63. Recently, p63 is considered to play a primary role in maintaining stem cell or reverse cell populations in several epithelia (Di Como et al., 2002, Nylander et al., 2002) including prostate epithelia (Signoretti et al., 2000). The expression of p63 has been identified in a variety of normal epithelial cells, including basal and suprabasal cells of stratified epitelia of skin, oesophagus, uterine cervix, tonsil and bladder, and basal cells of glandular structures of the bronchi, breast and prostate. It has been also discovered that p63<sup>-/-</sup> mice have severe defects in stratified epithelia, which causes newborn lethality, and lack organs arising from epidermis such as mammary and salivary glands (Mills et al., 1999; Yang et al., 1999). In the prostate, p63 is expressed in the basal cells (Yang et al., 1998). Signoretti et al. proposed that p63 is the stem cell factor for prostate because prostate development is absent in embryonic/newborn p63<sup>-/-</sup> mice (Signoretti et al., 2000).

The alteration of p63 expression might have impact in cancer stem cell regulation and cancer progression (Carrol et al., 2006; Boldrup et al., 2007; Dhillon et al., 2009). We found strong association between ALDH1A1 and cytoplasmic p63 expression. Based on our knowledge, no study has been done to reveal the association between ALDH1A1 and the ectopic expression of p63. However, consistent with our result, a study conducted by Carol et al., stated that p63 has been shown to positively regulate CD44 mRNA expression in microarray-based gene expression analyses of the MCF 10A immortalized nontumorigenic breast epithelial cell line. This study demonstrated that ectopic expression of p63 leads to the upregulation of CD44 expression, whereas shRNA directed against p63 mRNA leads to loss of CD44 expression (Carrol et al., 2006). It has also been proven in squamous cell carcinoma of head and neck, up-regulation and differential splicing of CD44 occurred following over-expression of  $\Delta Np63\alpha$ , an isoform of p63, which may indicate role of p63 in the regulation of adhesion, metastasis and the cancer stem cell phenotype (Boldrup et al., 2007). In addition, MCF7 mammospheres cells that expressed  $\Delta Np63\alpha$  showed some characteristics of cancer stem cell phenotype, including increase more colonies forming efficiency in soft agar, drug resistance, and tumorigenicity in transplanted NOD/SCID mice (Du et al., 2010). The knock-down of p63 has been proven to cause mammary cancer stem cells lose their self-renewal ability (Zucchi et al., 2008). Further study is needed to reveal how p63 may regulate cancer stem cell.

Our study showed the importance of cytoplasmic p63 expression was also demonstrated in prostate cancer progression, which difference in cytoplasmic p63 expression was found between benign lesion and prostate cancer, and between low and high Gleason score prostate cancer. Moreover, it is also found in our study that higher levels of cytoplasmic p63 were associated with a significantly higher frequency of proliferating cells and higher proportion of cells undergoing apoptosis. In agree with previous studies (except for apoptotic

rate), this research showed the altered expression of p63, a transcription factor involved in transactivation, apoptosis, and proliferation, may have potential oncogenic role (Narahashi et al., 2006; Dhillon et al., 2009). Interestingly, our result displayed a significant association of higher level of cytoplasmic p63 with higher apoptotic rate. Whereas previous study showed higher levels of cytoplasmic p63 was associated with lower apoptotic rate (Dhillon et al., 2009). This controversy made us searching for some literatures. We found, indeed, there is still controversy regarding the impact of p63 in apoptosis and differentiation. In stratified epithelia, the loss of normal p63 results in increased apoptosis (Senoo et al., 2007). Some studies showed  $\Delta Np63\alpha$  could caused cell cycle arrest and apoptosis, whereas another studies showed  $\Delta Np63\alpha$  could promote proliferation and inhibit apoptosis (Dohn et al., 2001). The controversy also exists regarding apoptotic role in prostate cancer progression. In study conducted by Aihara et al. and Dachille et al., significantly larger numbers of apoptotic bodies were observed in the areas of carcinoma than in the non-neoplastic control tissues. Moreover, a positive correlation was noted between apoptotic bodies and increasing Gleason score (Aihara et al., 1994, Dachille et al., 2008). Although our result displayed higher level of cytoplasmic p63 results in higher apoptotic rate, but this condition will lead to worse prognosis of patient since increased programmed cell death is a feature of the increasing malignant potential, which is associated with higher Gleason score in prostate cancer (Aihara et al., 1994). Unfortunately we found no significant association between apoptotic rate and Gleason score (data not shown).

In summary, although limited sample numbers and methods were used, but this research may contribute a brief description regarding ALDH1A1 and cytoplasmic p63 expression as a crucial components in prostate cancer progression. Different expression of those components in BPH, PIN, and different Gleason score of prostate cancer has been clearly demonstrated. ALDH1A1 and p63 alteration are suggested to have an important role in prostate cancer progression due to their association with pathological diagnosis, and, although not all of them, with cancer cell proliferation and apoptosis. The interaction between those components, although still clouded by controversy, needed to be outlined. Further study is demanded to reveal a clear mechanism on how cytoplasmic p63 and ALDH1A1 positive cells are taking role in cancer progression. If cancer stem cells and their regulating factors could be clearly identified, a new molecular marker and therapeutic agent for cancer might be developed.

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