

## RESEARCH ARTICLE

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## Loss of p27<sup>Kip1</sup> expression in high grade human prostate adenocarcinoma

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**ABSTRACT**

p27<sup>Kip1</sup> has been hypothesized to play a major role in carcinogenesis. Most of the published data reported that loss of p27<sup>Kip1</sup> expression was strongly associated with development and progression of tumour. The purpose of this study was to analyze p27<sup>Kip1</sup> expression in normal, benign and malignant prostate cancer tissues and their association with the clinicopathological parameters. The expression of p27<sup>Kip1</sup> was evaluated by an immunohistochemistry method. p27<sup>Kip1</sup> expression was significantly higher in normal and benign prostate tissues (P<0.01). In contrast, some malignant tissues had no p27<sup>Kip1</sup> expression and most had weak p27<sup>Kip1</sup> expression. p27<sup>Kip1</sup> expression was found to be decreased significantly with increasing Gleason scores (P=0.003). Most of prostate adenocarcinomas (PCa) with Gleason 8 and 9 showed loss of p27<sup>Kip1</sup> expression. The expression was also positively correlated with prostate specific antigen level and age in PCa group (P=0.003 and 0.043 respectively), whereas no association was found between the p27<sup>Kip1</sup> expression with tumour amount and age in benign prostatic hyperplasia group. This study suggests that loss of p27<sup>Kip1</sup> expression is essential during development and progression of prostate cancer.

**Key words:** carcinogenesis, prostate adenocarcinoma (PCa), benign prostatic hyperplasia (BPH), p27<sup>Kip1</sup>, immunohistochemistry**Introduction**

Prostate cancer is one of the most common cancers that occur in Malaysian male population and was ranked fourth among all human cancers prevalence. Prostate adenocarcinoma is the most common type of prostate cancer (National Cancer Registry, 2007) and its significance is related not only to the mortality, but also to its association to the high morbidity rate. Furthermore, there is no standard treatment that effectively cures this disease (Valicenti et al., 2000). Management of cancer patients including prostate cancer highly depends on accurate assessment of the biological potential of the tumour. Although the current assessment technique, which is focused on stage, grade and prostate specific antigen (PSA), is widely used, unfortunately, it is inadequate to determine the best treatment options to the patients. More precise marker is needed to aid

treatment decisions for prostate cancer patients.

It has been postulated that loss of negative cell cycle regulator might have a role in tumour development and progression (Sherr, 1996). One example of cell cycle regulator is p27<sup>Kip1</sup>. Loss of p27<sup>Kip1</sup> expression was seen in many human carcinomas like colorectal, mammary, esophageal, pulmonary and gastric (Loda et al., 1997; Tan et al., 1997; Catzavelos et al., 1997; Porter et al., 1997; Mori et al., 1997; Esposito et al., 1997; Singh et al., 1998). In addition, loss of p27<sup>Kip1</sup> expression was also found to be a negative prognostic marker in prostate carcinoma (Tsihlias et al., 1998; Yang et al., 1998; Cheville et al., 1998; Cordon-Cardo et al., 1998; Cote et al., 1998). p27<sup>Kip1</sup> is actually a protein that regulates the cell cycle transition from G<sub>1</sub> to S phase by binding to and inhibiting several cyclin dependent kinase (CDK) protein complexes in normal cells. Increased p27<sup>Kip1</sup> protein expression levels serves as a barrier for

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progression to S phase and signal exit from cell cycle, thus, block the cell proliferations (Polyak *et al.*, 1994). Lower levels of p27<sup>Kip1</sup> expression also could predict recurrent and poor disease-free survival in prostate cancer and is associated with other prognostic factors including higher tumour grade, positive surgical margins, seminal vesicle involvement, and lymph node metastasis (Dong, 2006).

In the present study, we conducted an immunohistochemical analysis to evaluate p27<sup>Kip1</sup> protein expression in normal, BPH and prostate adenocarcinoma. The possible association between p27<sup>Kip1</sup> expressions with clinicopathological parameters has also been assessed.

**Materials and Methods****Samples**

263 paraffin embedded specimens collected between 2006 and 2008 were obtained from the archive of Pathology Department in Hospital Kuala Lumpur. The patients' age ranged from 28 to 91 years (mean age, 64.54 ± 10.8 years). The specimens were composed of 63 normal prostate, 100 BPH and 100 PCa tissues. All the PCa cases were classified according to the Gleason grading system and were further divided into low score group (Gleason 6 to 7) and high score group (Gleason 8 to 9). The tumour volumes were classified as low amount, ≤ 5% and high amount, > 5%. Pretreatment PSA were grouped as either ≤ 4.0 ng/ml or > 4.0 ng/ml. Ethical approvals were obtained from the ethical committee of University Putra Malaysia (UPM), National Medical Research Registration (NMRR) and Hospital Kuala Lumpur (HKL) prior to commencement of the study.

**Immunohistochemistry**

p27<sup>Kip1</sup> immunohistochemical staining was done using the DAKO REAL EnVision (Dako, Ca, USA). A total of 263 formalin-fixed paraffin embedded tissues samples were processed. The paraffin blocks were cut at 4 µm, mounted onto poly-L-lysine glass slides and dried overnight at room temperature. Sections were dewaxed with absolute xylene and rehydrated with grading alcohol and lastly run under tap water. The sections then were heated in citrate buffer (0.01 M, pH 6.0) in 1000 W microwave oven for 10 minutes high temperature (98°C) followed by 10 minutes medium low temperature (72°C). After cooled down at room temperature for 20 minutes, endogenous peroxidase activity was inactivated in 3 % H<sub>2</sub>O<sub>2</sub> for 5 minutes. Then, the sections were rinse twice with Tris Buffered Saline (TBS) for 5

minutes. Monoclonal mouse anti-human p27<sup>Kip1</sup> (clone SX53G8, Dako, Ca, USA) were used at 1:50 dilutions in antibody diluents (Dako, Ca, USA). Sections were incubated with primary antibody for 1 hour at room temperature. After 1 hour, all sections were rinsed twice with TBS for 5 minutes before incubated with secondary antibody (Dako, Ca, USA) for 30 minutes. The sections were rinsed twice with TBS for 5 minutes. Diaminobenzidine (Dako, Ca, USA) was used as a chromogen to verify immunoreactions and hematoxylin was used for counterstaining. Negative control was processed simultaneously from the same samples and protocol but primary antibody step was replaced by antibody diluents. Colon adenocarcinoma sections were used as a positive control because this tissue was well known to express p27<sup>Kip1</sup>.

**p27<sup>Kip1</sup> expression analysis**

p27<sup>Kip1</sup> expression was evaluated by two blinded pathologists. All nuclear staining, either weak or strong were considered as a positive and no staining detected was considered as a negative. The expressions were scored from 0 to 4+ depending on the staining intensity. Quantification of p27<sup>Kip1</sup> immunohistochemical expression score was done using five randomly ocular fields under 200x microscope magnification, where 100 cells were counted in each field. The extent and intensity of positive tumour cells were graded as 0, none; 1+, weak; 2+, moderate; 3+, strong; and 4+, very strong (Yoshimura *et al.*, 2000). All the slides were evaluated without any knowledge of the patient's clinical data and histopathological reports.

**Statistical analysis**

Data were recorded and statistically analyzed using SPSS for Windows Version 17.0 (Chicago, USA) and the statistical results were considered significant for *P* values less than 0.05. Mann-Whitney test was used to compare the expression of p27<sup>Kip1</sup> between normal, benign, and malignant prostate tissues. The Chi-square test was used to determine the association of clinicopathological variables and p27<sup>Kip1</sup> expressions.

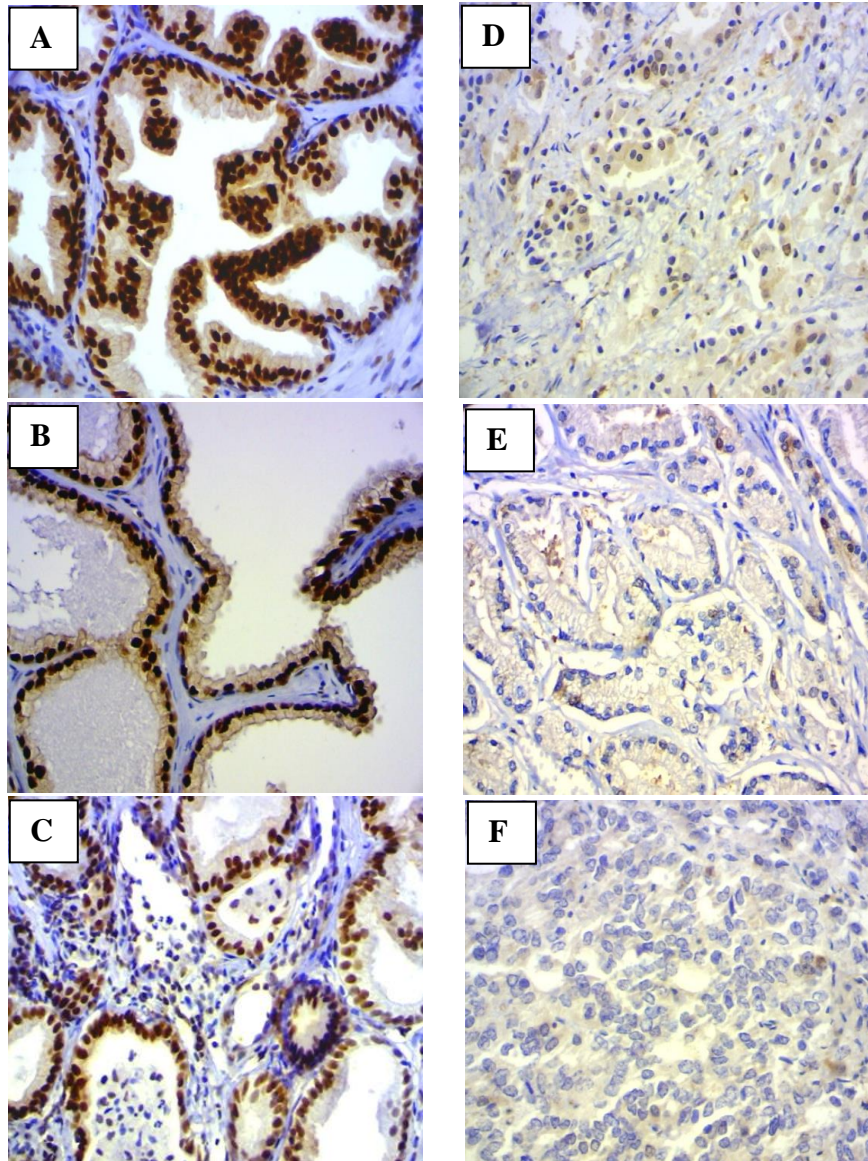
**Results**

p27<sup>Kip1</sup> was expressed in varying degrees of intensity by all the normal, BPH and PCa tissues. In this study, most of the immunoreactivity was detected in the nucleus of the epithelial cells surrounding the acini and occasionally in the stromal cells. In normal prostate tissues, the level and intensity of p27<sup>Kip1</sup> staining were uniformly high and the

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staining was concentrated within the nucleus but diffused cytoplasmic staining was also observed. 62 of 63 (98.4%) normal prostate tissues strongly expressed p27<sup>Kip1</sup> as shown in Figure 1A and Table 1. There was no negative or weak staining observed in the normal prostate tissues. Similar staining pattern was seen in BPH, where p27<sup>Kip1</sup> was strongly expressed in 77 of 100 (77%) (Figure 1B). In contrast, only

36 (36%) and 17 (17%) of PCa cases showed strong and moderate p27<sup>Kip1</sup> expressions respectively. The remaining 22 (22%) of PCa showed weak expressions and 25 (25%) were negative. Low and undetectable p27<sup>Kip1</sup> expressions were observed in the majority of high grade PCa tissues (Figure 1 D-F).



**Figure 1.** p27<sup>Kip1</sup> expressions in the nuclei of the acinar epithelial cells of the normal prostate, BPH, and PCa. A: Normal prostate shows very strong p27<sup>Kip1</sup> staining. B: BPH shows strong p27<sup>Kip1</sup> staining. C: PCa Gleason 6 (3+3) shows moderate to strong p27<sup>Kip1</sup> staining. D: PCa Gleason 7 (3+4) shows negative to weak p27<sup>Kip1</sup> staining. E: PCa Gleason 8 (4+4) shows negative p27<sup>Kip1</sup> staining. F: PCa Gleason 9 (4+5) shows negative p27<sup>Kip1</sup> staining. All samples were counterstained with hematoxylin. Original magnification is x200.

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**Table 1.** *p27<sup>Kip1</sup> expression in the normal prostate, BPH, and PCa*

| Tissue | Distribution of p27 <sup>Kip1</sup> staining intensity (%) |            |            |            |            | Total | P      |
|--------|--|------------|------------|------------|------------|-------|--------|
|        | 0  | 1+         | 2+         | 3+         | 4+         |       |        |
| Normal | 0 (0%)   | 0 (0%)     | 1 (1.6%)   | 27 (42.9%) | 35 (55.5%) | 63    |        |
| BPH    | 3 (3.0%)   | 4 (4.0%)   | 16 (16.0%) | 60 (60.0%) | 17 (17.0%) | 100   | 0.000* |
| PCa    | 25 (25.0%)   | 22 (22.0%) | 17 (17.0%) | 26 (26.0%) | 10 (10.0%) | 100   | 0.000* |

\* The difference of p27<sup>Kip1</sup> expressions between groups was significant at P<0.05.

**Table 2.** *Correlation between p27<sup>Kip1</sup> expressions with the clinicopathological parameters*

| Clinicopathological parameter            | p27 <sup>Kip1</sup> score |            | Total | P      |
|--|---------------------------|------------|-------|--------|
|  | 0-2 (Low)                 | 3-4 (High) |       |        |
| Patient's age in BPH (mean = 68.13±7.42) |                           |            |       |        |
| ≤68                                      | 10                        | 30         | 40    | 0.287  |
| >68                                      | 13                        | 47         | 60    |        |
| Patient's age in PCa (mean = 66.78±6.23) |                           |            |       |        |
| ≤66                                      | 22                        | 18         | 40    | 0.043* |
| >66                                      | 42                        | 18         | 60    |        |
| PSA level in PCa (ng/ml)                 |                           |            |       |        |
| ≤4.0                                     | 12                        | 17         | 29    | 0.003* |
| >4.0                                     | 52                        | 19         | 71    |        |
| Gleason score (tumour stage)             |                           |            |       |        |
| ≤7                                       | 41                        | 33         | 74    | 0.003* |
| >7                                       | 23                        | 3          | 26    |        |
| Tumour amount (%)                        |                           |            |       |        |
| ≤5                                       | 18                        | 17         | 35    | 0.055  |
| >5                                       | 46                        | 19         | 65    |        |

\*The association between p27<sup>Kip1</sup> expressions and clinicopathological parameters was significant at P<0.05.

The association between p27<sup>Kip1</sup> expressions with the clinicopathological parameters such as patients' age, PSA level, Gleason score and tumour amount were also studied. The intensity of p27<sup>Kip1</sup> and all the parameters were grouped into certain cut point. Table 2 summarizes the data relating p27<sup>Kip1</sup> expressions to the clinicopathological parameters.

The cut point for the patient's age in BPH was set based on the mean age (68.13 ± 7.42). There was no significant correlation between p27<sup>Kip1</sup> expressions and age group in BPH (P=0.287). In contrast, in PCa (mean age = 66.78±0.62), the patient's age was correlated to p27<sup>Kip1</sup> expression (P=0.043). In addition, there was a significant correlation of p27<sup>Kip1</sup> expressions between PSA levels of PCa patients (P=0.003) and Gleason scores (P=0.003). Tumour amount failed to correlate to p27<sup>Kip1</sup> expressions (P=0.055).

## Discussion

p27<sup>Kip1</sup> down-regulation has been postulated as a key event, which determination can be a strong prognostic marker, or can be useful for differential diagnosis in several tumour models. In this study, a significant down-regulation of p27<sup>Kip1</sup> protein expressions was detected in more than half of the malignant cases compared to the benign and normal prostate tissues. In accordance with other studies, a significant loss or down-regulation of p27<sup>Kip1</sup> expressions was widely found in PCa and the protein was significantly higher in BPH and normal prostate (Guo *et al.*, 1997; Zheng *et al.*, 2004; Nikloveshivili *et al.*, 2008). Our findings clearly showed high p27<sup>Kip1</sup> expressions in the normal prostate and BPH tissue. Only a small percentage (10%) of the prostate tumour tissues expressed high p27<sup>Kip1</sup>.

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The immunohistochemical analysis showed that the higher Gleason score had the greater total loss of p27<sup>Kip1</sup> expression (P=0.003). Gleason 9 cancer cells were totally negative for p27<sup>Kip1</sup> staining. This finding was in agreement with the previous studies as p27<sup>Kip1</sup> expression was reported to be either absent or reduced in high grade prostate cancer (Guo *et al.*, 1997; Tsihlias *et al.*, 1998; Cheville *et al.*, 1998; Erdamar *et al.*, 1999; Dreher *et al.*, 2004). This indicates that p27<sup>Kip1</sup> expression decreases when the initial steps of malignant transformation take place and suggested that when the neoplasm reaches the invasive stage, down-regulation is either maintained or increased. In addition, p38 encoded by *Jab1* gene interacts specifically with p27<sup>Kip1</sup> and over-expression of p38 can result in translocation of p27<sup>Kip1</sup> from the nucleus to the cytoplasm, causing a decreased level of p27<sup>Kip1</sup> in the cell by inducing its degradation through the ubiquitin/proteasome pathway (Pagano *et al.*, 1995). Therefore, p38 acts as a negative regulator to p27<sup>Kip1</sup>, and their specific binding may explain the regulation of cell cycle dependent proteolytic machinery and the selection of key cell cycle regulators for degradation (Tomoda *et al.*, 1999).

Chi-squared analysis was used to determine the association between p27<sup>Kip1</sup> expression and the clinicopathological parameters. PSA level in PCa patients (P=0.003), Gleason score (P=0.003) and patient's age in PCa (P=0.043) correlated with low p27<sup>Kip1</sup> expressions. Low expression of p27<sup>Kip1</sup> associated with elevated PSA level, increasing Gleason score and increasing age in PCa patients were similar with the study conducted by Nikoleishvili *et al.* (2008) and Halvorsen *et al.* (2003). Tumour amount (P=0.055) and patient's age in BPH (P=0.287) does not correlate with the expression of the p27<sup>Kip1</sup> protein. Halvorsen *et al.* (2003) also reported that p27<sup>Kip1</sup> expression was of borderline significant (P=0.054) associated with tumour amount as similar to this study. This result may be due to different methodologies, sample size, and cut points used to define p27<sup>Kip1</sup> expression in this study as compared to other studies (Drobnjak *et al.*, 2003).

p27<sup>Kip1</sup> is rarely mutated in human tumours including localized prostate carcinoma, however the homozygous deletion involving p27<sup>Kip1</sup> locus or loss of p27<sup>Kip1</sup> expression raised the possibility that genetic alteration of this gene may play an important role in prostate carcinoma (Kibel *et al.*, 2003). Guo *et al.* (1997) found that reduced p27<sup>Kip1</sup> expression correlated to high proliferative index and tumour grade. They also found that 4 of 5 metastatic samples demonstrated decreased staining intensity. Cordon-Cardo *et*

*al.* (1998) demonstrated low expression of p27<sup>Kip1</sup> in 83% of metastatic samples. Other studies found that decreased p27<sup>Kip1</sup> expression does not only correlate with decreased disease-free survival, but also overall survival (Cote *et al.*, 1998). In addition, Cheville *et al.* (1998) found that decreased p27<sup>Kip1</sup> expression correlated with adverse pathological features. Besides that, animal based studies demonstrated that loss of p27<sup>Kip1</sup> correlated with loss of Nkx3-1 in mouse prostate carcinogenesis (Gary *et al.*, 2004). Although genetic alteration of CDK inhibitor in prostate carcinoma are rare, but it still occur in certain cases because translational control is primarily responsible for the regulation of p27<sup>Kip1</sup> during cell cycle progression in response to TGF- $\beta$  (Hengst & Reed, 1996). Tomoda *et al.* (1999) reported substitution of glycine for valine in p27<sup>Kip1</sup> polymorphism, causing p38<sup>Jab1</sup> to promote the phosphorylation and cytoplasmic translocation of p27<sup>Kip1</sup> for its subsequent degradation in the cytoplasm, therefore lower the level of p27<sup>Kip1</sup> in the nucleus. Kibel & Isaacs (2000) also conclude that p27<sup>Kip1</sup> is the strongest candidate as a biomarker. They further mentioned that increased p27<sup>Kip1</sup> expression appears to correlate with improved pathological features and disease-free survival.

In addition, several functions have been described to a p27<sup>Kip1</sup> protein, but loss of its expression is thought to be tumourigenic and may lead to tumour progression. Other than those functions, p27<sup>Kip1</sup> is also involved in apoptosis promotion, regulation of drugs resistance in solid tumours and have protective role in inflammatory injury (Ponce-Castaneda *et al.*, 1995). The fact that loss of p27<sup>Kip1</sup> expressions in mice significantly affects tumour progression, in conjunction with the observation that its protein level is reduced in human tumour suggests that restoring p27<sup>Kip1</sup> may be beneficial in prostate cancer therapy (Blain *et al.*, 2003).

**Conclusion**

In conclusion, we suggest that p27<sup>Kip1</sup> might have a significant role in prostate cancer development and progression. There are powerful arguments and suggestions that p27<sup>Kip1</sup> should be evaluated further as one of the potential therapeutic targets for prostate cancer or other types of cancer in Malaysia. Additional studies are required to examine the potential use of p27<sup>Kip1</sup> as a prognostic marker to strengthen the evidences for better aggressive screening, prophylaxis, and treatment of prostate cancer.

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