Research Article

Can cyclin D1 be utilized as a second step "after basal cell marker" for both diagnosis and prognosis of prostatic adenocarcinoma?

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Abstract

Background: Cyclin D1 is involved in regulating cell cycle as well as cancer progression and its overexpression is incriminated in the pathogenesis of many tumors including prostatic adenocarcinoma (PAC). One of the basal cell markers is p63. p63 is a nuclear protein, which plays a critical role in the growth and development of many epithelial organs. p63 is confined to basal cells of squamous epithelia and urothelium, as well as basal cells of many organs including, prostate. So, confirmation of the presence or absence of basal cell layer is the first clue to deny or ascertain the diagnosis of PAC.

Objective: To investigate the expression of cyclin D1 with p63 in both benign prostatic hyperplasia (BPH) and PAC and correlate the results of cyclin D1 with both Gleason 's score and clinical staging.

Materials and Methods: Retrospective evaluation of archived specimens of BPH and PAC and p63 was used for more confirmation of the histopathological state. This study was done in 60 cases of BPH and 60 cases of PAC. All cases were stained for p63 before staining with cyclin D1 antibody. All immunohistochemical analyses were performed on routinely processed, formalin-fixed, paraffin-embedded tissue. Any degree of reactivity was considered positive. Percentage of positive cells was calculated and scored as follows: + (weak) = less than 10%, ++ (moderate) = 11% to 50%, and +++ (strong) = more than 50% tumor cells stained positive. Immunohistochemical expressions were correlated with Gleason grade as well as staging of PAC in an attempt to recognize the prognostic value of cyclin D1.

Result: Cyclin D1 was expressed in 13% and 93% of BPH and PAC cases, respectively, opposite to 92% and 4% in that of p63. In PAC cases, the degree of cyclin D1 reactivity was increased in high Gleason's grade with significant correlation ($P = 0.043$). Also a significant correlation was obtained between the degree of staining and the staging of PAC ($P = 0.045$).

Conclusion: Cyclin D1 is highly expressed in PAC cases and its overexpression was seen in high Gleason's score and staging beyond T2.

KEY WORDS: Benign prostatic hyperplasia, cyclin D1, immunohistochemistry, prostatic adenocarcinoma, p63

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Introduction

Cyclin D1 is articulated in the G1 phase of the cell cycle and has a vital role in regulating the cell cycle and also in cancer progression. Its overexpression resulting from gene amplification is believed to participate in both the pathogenesis and grading of many tumors including prostatic adenocarcinoma (PAC).[1] In PAC, cyclin D1 acts as a critical

regulator of androgen-dependent transcription and cell cycle progression.[2]

Cyclin D1 expression in a variety of tumors was studied for genetic valuation and some signals were expounded from these studies to hypothesize that amplification of the 11q13 region is tangled in a variety of human tumors[3] including bladder carcinoma, head and neck squamous cell carcinoma, and carcinomas of the esophagus and breast.[4] In the amplified 11q13 region, several genes have been branded, of which cyclin D1 and p27 are the most consistently amplified and overexpressed.[5,6]

Cyclin D1 amplification and consequently overexpression of its protein "cyclin D1" envisage poor outcome as evidenced by a study by Seiler et al.[7] who studied this amplification on bladder cancer and revealed that patients with this genetic flaw have a twofold probability of dying from cancer bladder compared with patients without this defect.

The discovery of p63 as a basal cell marker makes it a useful stain for diagnosis of conflicting cases of prostatic lesions, especially when it is associated with one of the growth regulating factors as cyclin D1.[8]

p63, a p53-homologue nuclear transcription factor that is located on 3q27-29 and encodes six different isoforms, which harbor either transactivating or negative dominant effects on p53 reporter genes.[8,9] It plays a critical role in the growth and development of many epithelial organs and is confined to basal cells of squamous epithelia (including epidermis and hair follicles) and urothelium, as well as basal cells/ myoepithelial cells in breast, sweat glands, salivary glands, and prostate.[9]

The objectives of the presenting study were to investigate the expression of cyclin D1 in both BPH and PAC and correlate the results of cyclin D1 with the Gleason's score as well as clinical staging of PAC. The use of p63 in our study was to confirm the presence or absence of basal cell layer, and accordingly, the precise confirmative diagnosis will be more established.

Materials and Methods

This study was carried out in 120 prostatic specimens distributed equally between BPH and PAC (60 for each). Cases of PAC were of different Gleason's score. Sample dealings were different and they included transurethral resection prostatectomy (78 cases) and radical prostatectomy (42 cases). Of the radical prostatectomy, 40 cases were diagnosed as PAC and consequently, these cases were applied for staging. Clinical data were obtained from the patient's files and referral forms. The clinical data included age, clinical presentation, and prostatic specific antigen (PSA) measurement, if available.

For histopathological examination, tissue samples were routinely fixed in 10% formalin, embedded in paraffin, cut into 4-mm thick sections, and stained with hematoxylin and eosin stain. In PAC cases, each case was graded according to the International Society of Urological Pathology modified Gleason grading system[10] and distributed according to their Gleason's score into two main groups: group I (Gleason's score ≤7) and group II (Gleason's score >7). The staging was applied in 40 cases that were obtained by radical prostatectomy specimens. The staging was applied according to American Joint Committee on Cancer definition of the primary tumor,[11] in which cases of PAC were distributed according to their pathological stage into two main groups: organ confined (<T2) or extended outside the capsule (>T2).

For immunohistochemical staining; all immunohistochemical (IHC) analyses were performed on routinely processed, formalin-fixed, and paraffin-embedded tissue. Tissue sections were cut at 4 μm and mounted on poly-l-lysine-coated slides.

Regarding cyclin D1, immunohistochemical staining was performed with monoclonal anti-cyclin D1 antibody (anti-cyclin D1 antibodies, clone 2D11F11, 1:20; Novocastra Laboratories/ Vector Laboratories, Burlingame, CA, USA), at a dilution of 1:20, using a standard avidin/biotin complex (ABC) method. The automated staining procedure consisted of a 45-min incubation with the primary antibody, followed by brief buffer washes, and then incubation in a cocktail of biotinylated anti-mouse IgG/IgM (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 min.[12] The slides were then washed, incubated in ABC (Sincere Biotech, Shunyi, China) for 30 min, washed, and then reacted with diaminobenzidine and hydrogen peroxide to visualize the end product. The sections were counterstained with hematoxylin.

Regarding p63 immunostaining, immunostaining was performed in all tissue specimens using the 4A4 anti-p63 antibody (Dako, Denmark, Code M7247). The antibody was diluted in the ratio 1:50. The following steps were done: 4-μm cut sections were deparaffinized, rehydrated, and subjected to microwaving in 10 mmol/L citrate buffer at pH 6.0 in a 750 Woven for 15 min. Slides were left for cooling at room temperature for 30 min. The diluted antibody was applied at room temperature for 2 hours in an automated stainer (Optimax plus 2.0 bc; BioGenex, San Ramon, CA, USA). Detection steps were performed by the instrument using the Multi-Link-HRP kit (BioGenex, San Roman, CA, USA). Peroxidase activity was localized using 3, 3-diaminobenzidine-nickel chloride. Standardized development time periods allowed accurate comparison of all samples.

Evaluation of staining reactivity for both markers was done and the only discrete nuclear staining of tumor cells was considered positive. Any degree of nuclear staining was considered positive. The percentage of positive cells was then calculated and staining categories were graded as follows: + (weak) = less than 10%, ++ (moderate) = 11% to 50%, and +++ (strong) = more than 50% tumor cells stained positive.[2,13]

Statistical analysis was done by using χ^2test of independence and the Fisher's exact test to evaluate the significance of association between different grades of reactivity of both markers in BPH and PAC with other clinicopathological values and to correlate the reactivity data in PAC cases with Gleason's score and staging. The degree of agreement between cyclin D1 and p63 expression was measured by the Kappa measure of agreement. The Student's t test was used to estimate the

difference in quantitative variables. For quantitative variables, mean (as a measure of central tendency), the standard deviation, and minimum and maximum (as a measure of variability) were estimated. Frequency and percentage were presented for qualitative variables." All *P*-values were two sided. *P*-values less than or equal to 0.05 were considered significant. Computer software Statistical Package for the Social Science (version 17) was used in the analysis of the data presented.

The sensitivity and specificity tests were done for both markers in BPH and PAC cases by using the following formulas:

Sensitivity = True Positive (a)/True Positive + False Negative (b) × 100%

Specificity = True Negative (d)/True Negative + False Positive (c) × 100%.

Result

Regarding clinical data, the age of selected cases ranged from 51 to 92 years, age of PBH cases ranged from 48 to 74 years with a mean of 66 ± 3.7 years, whereas age of PAC cases ranged from 55 to 84 years with a mean age of 71 ± 2.3 years.

Level of PSA in BPH ranged from 2 to 5 ng/mL with the mean value 2.5 ± 1 ng/mL, in comparison to 7–84 ng/mL with a mean value of 34 ± 4 ng/mL in PAC cases.

In this study, all clinical presentation was in the form of lower urinary tract obstructive symptoms such as dysuria, hesitancy, and urgency as reported in the patient's file.

Regarding PAC staging, all cases were distributed into two main groups: group I (<T2): (*n* = 22; 55%) and group II (≥T2): (*n* = 18; 45%). According to the results of *t* test, there is no significant correlation between clinical data and the results of either p63 or cyclin D1 (*P* = 0.621 and *P* = 0.512, respectively).

Histopathological reviewing examination revealed that 60 cases were diagnosed as BPH and 60 cases were diagnosed as PAC. All cases of PAC were divided according to Gleason's score into two main groups: group I (40 cases; 66.6%), group II (20 cases; 33.4%)

Results of p63 reactivity showed that cases of BPH revealed positivity in 56 cases out of 60 (93%). Of the positive cases, the following results were obtained: focal weak staining (*n* = 2; 3%), moderate (*n* = 14; 23%), and diffuse strong staining (*n* = 40; 67%) [Table 1, Figure 1]. In PAC cases, negativity was observed in 58 cases out of 60 (97%), whereas weak positivity was seen in two (3%). According to the results of the χ^2 test, there is no significant correlation between Gleason's score and the results of p63 staining (*P* = 0.528). Few foci of prostatic intraepithelial neoplasia (PIN) associated with PACs showed positive p63 staining.

According to the results of *t* test, no significant correlation was obtained between PSA and reactivity of p63 staining in both BPH and PAC cases (*P* = 0.621 and 0.581, respectively). Sensitivity and specificity of p63 for BPH cases were 93.33% and 96.67%, respectively.

Results of cyclin D1 reactivity showed that cases of BPH revealed positivity in eight cases (13%). Of the positive cases, the following results were obtained: weak positivity (*n* = 6; 10%), moderate (*n* = 1; 1.5%), and strong positivity (*n* = 1; 1.5%) (Table 1, Figures 2, 3 and Graph 1).

Cases of PAC revealed positivity in 56 of 60 cases (93%). Of the positive cases, the following results were obtained: weak positivity (*n* = 12; 20%), moderate (*n* = 23; 38%), and strong positivity (*n* = 21; 35%) (Table 1, Figures 4,5,6,7 and Graph 1).

Cyclin D1 results in relation to Gleason's score revealed that in group I (Gleason's score ≤7, *n* = 40 cases), the following results were obtained: negativity (*n* = 4 cases; 10%), weak positivity (*n* = 10 cases; 25%), moderate (n = 14 cases;

Table 1: Results of p63 and cyclin D1 reactivity in BPH and PAC cases

IHC stain	Lesion	Number of cases	Degree and percentage of p63 and cyclin D1 reactivity			
			Negative	Weak (+)	Moderate (++)	Strong (+++)
p63	BPH	60	4 (7)	2 (3)	14 (23)	40 (67)
	PAC	60	58 (97)	2 (3)	0	0
Cyclin D1	BPH	60	52 (87)	6 (10)	1 (1.5)	1 (1.5)
	PAC	60	4 (7)	12 (20)	23 (38)	21 (35)

BPH, benign prostatic hyperplasia; PAC, prostatic adenocarcinoma; immunohistochemistry (IHC)

Table2: Relation between the results of cyclin D1 reactivity and Gleason's score in PAC cases

Gleason's score	Number	Number and percentage of cyclin D1 reactivity			
		Negative	Focal	Moderate	Strong
Group I: ≤7	40	4 (10)	10 (25)	14 (35)	12 (30)
Group II: >7	20	0 (0)	2 (10)	9 (45)	9 (45)

PAC, prostatic adenocarcinoma.

Table3: Relation between the results of cyclin D1 reactivity and staging in PAC cases

Stage	No.	Reactivity of PAC cases to cyclin D1			
		Negative	Weak	Moderate	Strong
<T2	18	2	4	7	5
>T2	22	1	2	6	13
Total	40	3	6	13	18

PAC, prostatic adenocarcinoma.

35%), and strong positivity (n = 12 cases; 30%). In group II (Gleason's score >7, n = 20 cases), the following results were seen: negativity (n = 0 case; 0%), weak positivity (n = 2 cases; 10%), moderate (n = 9 cases; 45%), and strong positivity (n = 9 cases; 45%) (Table 2, Figures 4, 8 and Graph 2].

According to the results of χ^2 test, a significant correlation was obtained between Gleason's score and the degree of staining reactivity with P = 0.043.

Staging and Staining Reactivity

PACs in stage <T2 (n = 18 cases) showed negativity (n = 2 cases), weak positivity (n = 4 cases), moderate positivity (n = 7 cases), and strong positivity (n = 5 cases). Cases of PAC in stage ≥T2 showed negativity (n = 1 case), weak positivity (n = 2 cases), moderate (n = 6 cases), and strong positivity (n = 13 cases) (Table 3 and Graph 3). Application of the χ^2 test revealed a significant correlation between the staining results of both PAC staging groups (P = 0.045).

According to the results of t test, there is no significant correlation between cyclin D1 expression and PSA in both BPH and PAC cases with P = 0.564 and 0.484, respectively. Sensitivity and specificity of cyclin D1 for PAC cases were 93.33% and 86.67%, respectively. There are significant correlations obtained between the results of cyclin D1 and p63 in both BPH and PAC cases with p value = 0.045 and = 0.038, respectively.

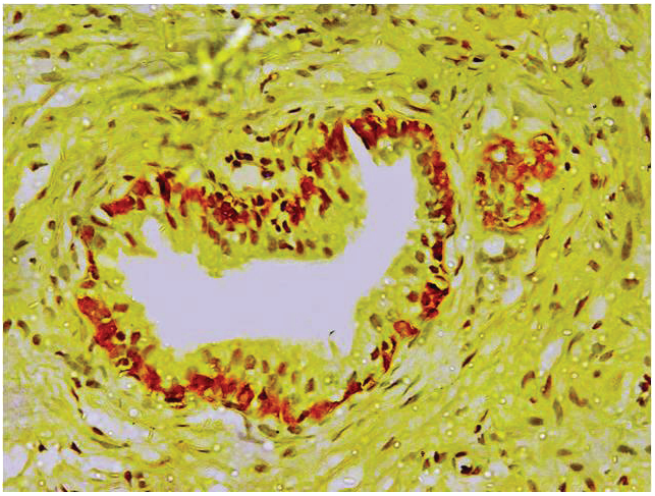


Figure 1: A case of BPH showing strong reactivity for p63 antibody stain (DAB) (x400) (BPH, benign prostatic hyperplasia; 3, 3-diaminobenzidine (DAB)).

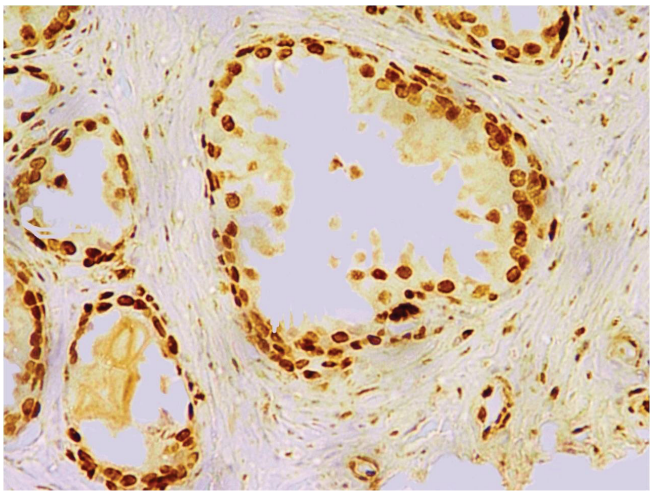


Figure 2: A case of BPH showing strong reactivity for cyclin D1 antibody stain (ABC) (DAB) (x400) (BPH, benign prostatic hyperplasia; ABC, avidin/biotin complex; 3, 3-diaminobenzidine (DAB)).

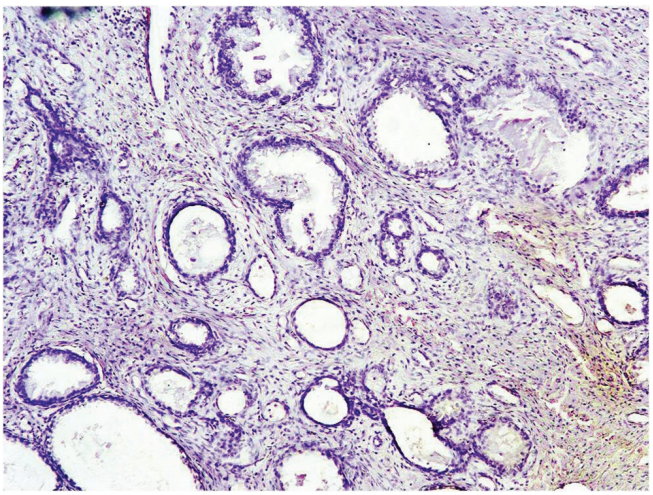


Figure 3: A case of BPH showing negative staining for cyclin D1 antibody stain (ABC) (DAB) (x200) (BPH, benign prostatic hyperplasia; ABC, avidin/biotin complex; 3, 3-diaminobenzidine (DAB)).

Discussion

According to the clinical results obtained, our results showed that the results of clinical data and PSA level were reliable with previous studies and no abnormalities were detected.[14,15]

According to p63 immunostaining, the positivity of basal cell layer in BPH and PAC cases were seen in 92% and 4% of cases, respectively. These results were consistent with many previous studies[9,16–18] as these results showed nuclear

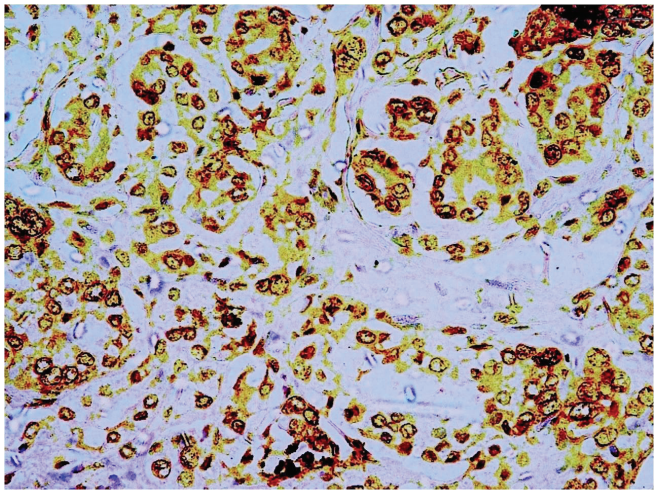


Figure 4: A case of PAC of Gleason pattern 2 showing strong positive staining for cyclin D1 antibody stain (ABC) (DAB) (x400) (PAC, prostatic adenocarcinoma; ABC, avidin/biotin complex; 3, 3-diaminobenzidine (DAB)).

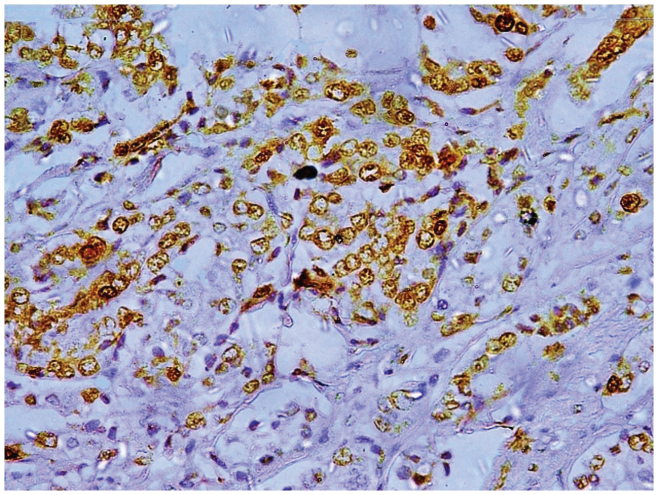


Figure 5: A case of PAC of Gleason pattern 3 showing strong positive staining for cyclin D1 antibody stain (ABC) (DAB) (x400) (PAC, prostatic adenocarcinoma; ABC, avidin/biotin complex; 3, 3-diaminobenzidine (DAB)).

positivity of basal cell layer of prostatic acini in BPH cases as well as negativity of secretory cells for p63 staining. The results obtained from these studies for BPH cases revealed high positivity of basal cell layer that ranged from 90% to 100%. In addition, these studies confirmed the high negativity of PAC cases to p63 that reached to be 97% to 100%. The sensitivity and specificity of p63 for basal cell identification were 93.3% and 96%, respectively; this coincides with that of Ng et al.[16] who revealed it to be 83.37% and 100%, in that order.

Romics et al.[18] studied the expression of p63 besides p21 and androgen receptor proteins relative to serum PSA levels in normal prostate and PAC of different Gleason's score, and revealed that p63 and p21 proteins were detected in normal basal cell nuclei and were lost in PAC cases.

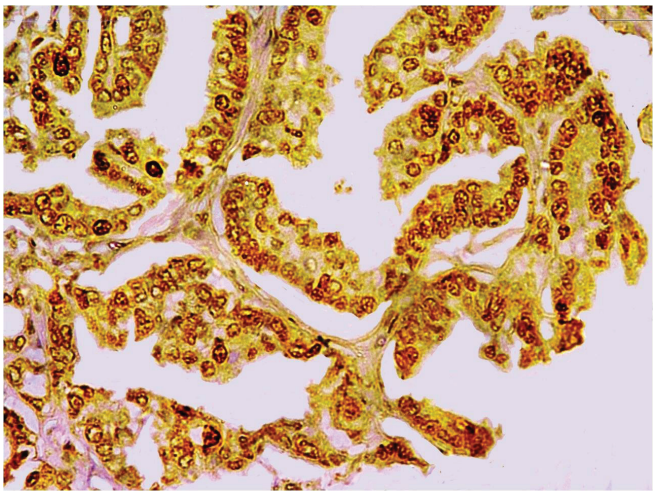


Figure 6: A case of PAC of Gleason pattern 4 (papillary pattern) showing strong reactivity for cyclin D1 antibody stain (ABC) (DAB) (x400) (PAC, prostatic adenocarcinoma; ABC, avidin/biotin complex; 3, 3-diaminobenzidine (DAB)).

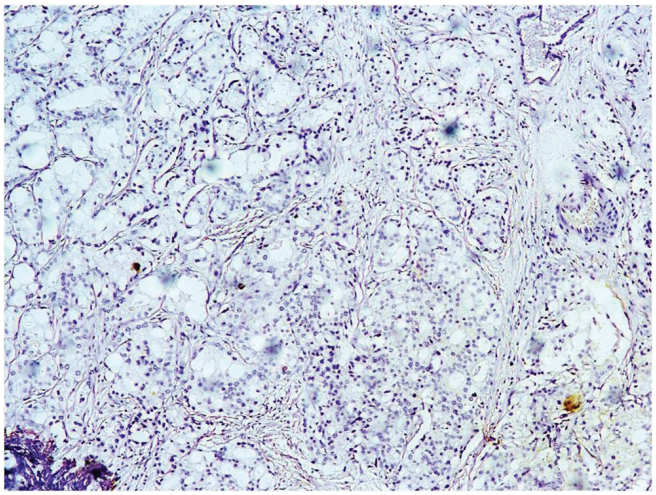
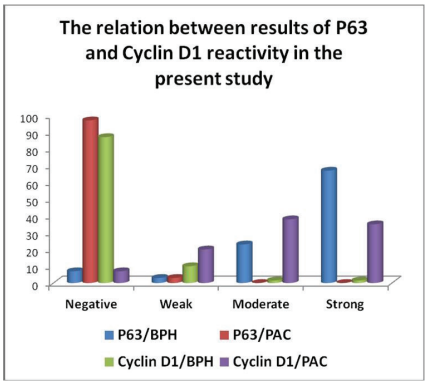


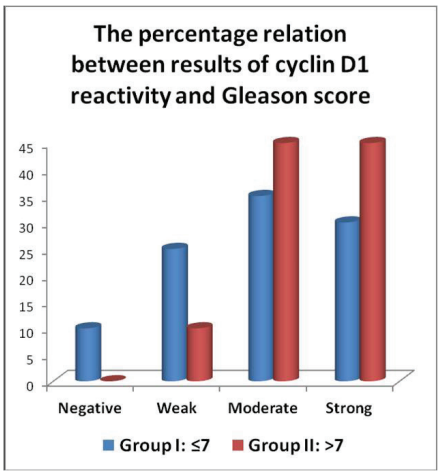
Figure 7: A case of PAC showing negativity for cyclin D1 antibody stain (x200) (PAC, prostatic adenocarcinoma).

In this study, we observed four cases of BPH (8%) that shIn the presenting study, we observed four cases of BPH (8%) that showed p63 negativity and focal positivity for cyclin D1, that is, absence of basal cell layer [Table 1]. This remark was also noted and reported in previous studies.[9,19] Shah et al.[19] explained this observation by the effects of prolonged formalin fixation that decreases the p63 antigenicity or the presence of cautery artifact or technical variabilities or the true absence of basal cells because of absent gene expression of basal cell markers.

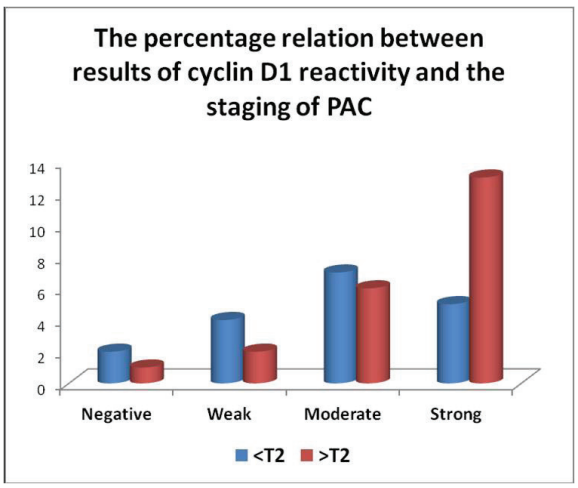
Our presenting study revealed two cases of PAC (4%) showing focal p63 positivity along with strong positivity to cyclin D1 were noticed. Also, this observation was noticed by Osunkoya et al.[20] who reported that prostate cancer can



Graph 1: Reactivity of both p63 and cyclin D1 in both BPH and PAC cases (BPH, benign prostatic hyperplasia; PAC, prostatic adenocarcinoma).



Graph 2: Reactivity of cyclin D1 in PAC cases in relation to Gleason's score (Group 1 ≤ 7, Group II > 7) (PAC, prostatic adenocarcinoma).



Graph 3: Reactivity of cyclin D1 in PAC cases in relation to tumor stage (PAC, prostatic adenocarcinoma).

aberrantly express diffused p63 staining in a nonbasal cellular distribution leading to the wrong diagnosis especially, in conflict cases.

Regarding cyclin D1 expression in this study, nuclear positivity only was considered irrespective of cytoplasmic positivity as done in some prior studies[13,21] in contrast to the other studies[22,23] in which both cytoplasmic and nuclear staining was considered. In this study, results obtained for cyclin D1 revealed wide variations toward BPH and PAC results.

Our results revealed that cyclin D1 expression was found only in 16% of BPH cases, this result is very different from the previous two studies in which cyclin D1 positivity was present in 72% and 70%,[24,25] respectively. On the other hand, our results showed that cyclin D1 was expressed in 56 cases out of 60 (93%). This result is close to the results obtained in the studies by Comstock et al.,[22] Fleischmann et al.,[24] and Pereira et al.[25] in which cyclin D1 positivity ranged from 100% to 75%. On the other hand, our results are contrary to the results by Kallakury et al.[21] and Drobnjak et al.[13] in which cyclin D1 positivity was seen in 11% and 22, respectively. Gumbiner et al.[26] revealed that 4.2% of the prostate tumors showed overexpressed cyclin D1 transcripts; however, normal levels of cyclin D1 were found in the BPH specimens examined.

Cheville et al.[27] evaluated p27 "a gene responsible for cyclin D1 expression" in 138 PACs treated by radical prostatectomy to assess its association with other numerous data, including Gleason's score and staging. They found that patients with PAC exhibited low p27 expression and had higher mean Gleason's scores than did high expressers with significant correlation. Also, they found a correlation between low p27 expression with positive surgical margins as well as lymph node metastasis.

In this study, cyclin D1 positivity in PAC cases revealed an increasing positivity of staining with high Gleason's score and significant correlation (*P*-value = 0.043). This significant correlation is in agreement with the studies done by Romics et al.[18] and Kallakury et al.[21] and contrary to studies by Zagars et al.,[11] Ud Din et al.,[17] and Shah et al.,[19] who did not find a significant correlation. This discrepancy may be attributed to the fact that most of the studies, including ours, focused largely on nuclear cyclin D1 positivity and ignored cytoplasmic study, a low percentage of positive cyclin D1 expression in PAC cases, and presence of mass variation in these studies.[12,21]

Han et al.[28] studied the expression of cyclin D1 in human PAC cell lines of 50 primary prostate cancer samples, and they revealed that cyclin D1 protein was highly expressed in all of the six human prostate cancer cell lines examined and 24% cases of PAC revealed regions of moderate to strong cyclin D1 positivity, but it was not discovered in the cultures of normal human prostate cells, which suggested that additional studies on the expression of this gene to understand the pathogenesis of PAC should be conducted.

In this study, a significant correlation was obtained between the results of cyclin D1 reactivity with the staging of PAC. This

is in agreement with the study by Drobnjak et al.[13] who found that cyclin D1 positive phenotype was found in 5.9% of pT2 cases compared with 35% of pT3 lesions, so the difference was marginally significant ($P = 0.045$). On the other hand, our result is in contrast to a recent study done by Seiler et al.[7] who found that cyclin D1 status in the primary tumors and in the metastases was not significantly related to the stage of the primary tumor or lymph node metastases.

It is well known that cyclin D1 is a well-established oncogene in many different cancers, including PAC.[29] The cyclin D1 gene and the expression of its protein product "cyclin D1" are frequently altered in human cancers and may predict tumor progression.[30] As per these data, a recent study was done by Elliman et al.[31] who studied the expression and role of a gene named, microRNA 206 (miR-206 gene) that regulates cyclin D1 expression and acts as a tumor suppressor gene. Elliman et al.[31] showed that this gene has a role in pathogenesis of some cancers, including prostate cancer and revealed that several PAC cell lines have lost miR-206 expression resulting in deregulated cyclin D1 expression that can contribute to their tumorigenic state.

Conclusion

Cyclin D1 (positive)/p63 (negative) might support the diagnosis of PAC, especially in conflicting cases. The positivity of cyclin D1 in PAC cases was increased in high Gleason's score and PAC of advanced clinical staging. Therefore, cyclin D1 overexpression in PAC cases might be used as a landmark for poor prognosis. So, the optimistic correlation between Gleason's score, staging of PAC, and cyclin D1 expression possibly must be used as a prognostic indicator in PAC. As a result, more researches must be focused toward the molecular analysis of cyclin D1 and the gene that regulates its expression in PAC as miR-206 gene to ascertain the proper way for the management of PAC.

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