RESEARCH ARTICLE

IMMUNOHISTOCHEMICAL EXPRESSION OF CYCLIN D1 AND KI 67 IN PREMALIGNANT AND MALIGNANT PROSTATIC LESIONS.
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Manuscript Info

Abstract

**Background:** Prostatic carcinoma is a common and growing public health problem. Cyclin D1 is an amino acid that is expressed in the cell cycle and has an important role in regulating cancer progression, also, The Ki-67 protein functions as a nuclear antigen that is only expressed in proliferating cells. It is a marker of the growth fraction in malignant tissue.

**Objectives:** to study the expression of Cyclin D1 and Ki-67 in prostatic adenocarcinoma and PIN and correlate their expression with age, preoperative serum PSA and Gleason score.

**Material and Methods:** A total of 50 cases including 25 cases of prostatic carcinoma, 10 cases of high grade PIN and 15 cases of BPH (as a control group) were studied. Tumor grade was determined according to Gleason’s grading system. Cyclin D1 and Ki-67 expression was determined by IHC staining.

**Results:** In carcinoma cases, Cyclin D1 was expressed in 23/25 cases(92%), in PIN it was expressed in 8 / 10 cases (80%) while in BPH it was expressed in only 3 /15 cases(20%). Ki-67 was expressed in 20 / 25 (80%) cancer cases, in PIN it was expressed in 7 / 10 (70%) cases while in BPH it was expressed in only 1 / 15 case (6.7%). Both Cyclin D1 and Ki-67 were significantly up-regulated in cancer cases as compared to BPH cases (P< 0.001). No significant correlation was found between the intensity of Cyclin D1 expression and Gleason score, whereas a statistically significant correlation was observed between the intensity of Ki 67 expression and Gleason score (P < 0.001). A statistically significant positive correlation was observed between Cyclin D1 and Ki-67 expression in cancer group, PIN group and BPH group with P value 0.03, 0.001 and 0.04 respectively.

**Conclusion:** Cyclin D1 may be helpful in differentiation between BPH, PIN and adenocarcinoma of the prostate, while Ki-67 can be applied along with other prostate cancer prognostic factors.

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Introduction:-
Prostatic diseases are responsible for significant morbidity and mortality in men all over the world (1). In men older than 50 years, prostatic carcinoma represents the most common malignant tumor and is characterized by a highly variable clinical outcome (2). It accounts for about 50% of male genital tract malignancies in Egypt (3), and prostatic intraepithelial neoplasia (PIN), is probably the most important precursor lesion of invasive prostatic cancer (4). Cyclin D1 has been identified as a short-lived nuclear protein involved in cell cycle transition from growth (G1) to synthesis (S) phase in both normal and neoplastic cells (5). The relationship between cyclin D1 expression and prostate cancer remains controversial. Some researchers have found occasional cyclin D1 expression in prostate cancer, whereas others reported that prostatic tumors with high cyclin D1 expression were associated with a more aggressive disease (6).

Ki-67 is a protein involved in cell cycle regulation and cell proliferation. It is expressed in proliferating cells during all active phases of the cell cycle (apart from G0). It is confirmed to be the most promising biomarker for routine practice applications (7), which may reflect the biologically malignant potential of prostatic carcinoma and justify its use in selecting candidates for active surveillance. However, its significance remains controversial (8).

Aim of the work:-
Because the role of cyclin D1 and Ki-67 in prostate cancer is unclear, we studied the expression of cyclin D1 and Ki-67 in PIN, prostatic adenocarcinoma and benign prostatic hyperplasia (BPH) (as a control group) to evaluate the relationship of these proteins with some clinicopathological features as age, preoperative serum PSA and Gleason score and to declare a possible correlation between Cyclin D1 and Ki-67.

Material and Methods:-
Specimens:-
Paraffin blocks prepared from 50 specimens of prostatic tissue were collected in this study, including: 7 radical prostatectomy specimens, 18 transurethral specimens (TUR), 22 transrectal needle biopsies (TRUS) and 3 cases of open prostatectomy. The specimens were obtained from the Department of Pathology, Faculty of Medicine, Cairo University, in the period from January 2013 to December 2014. The specimens included 25 cases of previously diagnosed prostatic carcinoma, 10 cases of high grade PIN and 15 cases of BPH (as a control group). For all cases, available clinical data such as age of the patients and preoperative serum PSA and Gleason score were evaluated.

Histopathological examination:-
Each paraffin block was cut into serial sections and stained by Hematoxylin and Eosin (H&E). In histopathological evaluation of the prostatic adenocarcinoma, Gleason grading system was used modified by Amin et al. (9).

Immunohistochemical staining:-
Immunohistochemical analysis was performed on routinely processed, formalin-fixed, paraffin-embedded tissue. Tissue sections were cut at 5 microns thickness, mounted on charged slides and stained by Cyclin D1 using Monoclonal Mouse anti-human Cyclin D1 (SPM587-NBP2-32840, Novus Biological, USA) and by Ki-67 using polyclonal Rabbit anti-human Ki-67(NB500-170, Novus Biological, USA).

After routine deparaffinization in xylene, the sections were hydrated through a series of graded alcohols, distilled water, and phosphate-buffered saline (PBS) at pH 7.2-7.4. Antigen retrieval was performed using Tris-EDTA (PH=9) at the microwave for Cyclin D1 and using citrate buffer (PH=6) for Ki67 immunostaining.

Blocking reagent (Ultra V Block) was placed on each slide and incubated for 10 minutes at room temperature in the humidity chamber. Excess serum was shaken off without washing. Each primary antibody was placed on the slides. The slides were incubated overnight at room temperature in humid chamber. The slides were rinsed for 5 minutes. 2-3 drops of secondary antibody (biotinylated polyvalent) diluted in this buffer solution TBS, were placed on each slide. The slides were incubated in a humidity chamber at room temperature for 15 minutes. After tapping off excess buffer the slides were rinsed for 5 minutes, twice, in PBS and were incubated in Streptavidin enzyme for 15 minutes at room temperature. The slides then rinsed for 5 minutes, twice, in PBS. Working Diaminoben zidine tetrychochloride (DAB) reagent was placed on each slide (approximately 500 ml per section). The slides were incubated for 4 minutes at room temperature in the humidity chamber and then washed thoroughly in distilled water. Counter stain with Mayer's hematoxyline for 1 minute was done then the slides were washed. The slides were placed...
in two changes of 95% ethyl alcohol, then two changes of absolute alcohol, each for two minutes. Finally, the slides were cleared in xylene and cover slips were fixed using mounting reagent. Each slide was evaluated by the authors.

**Evaluation of Cyclin D1 expression:**
Cyclin D1 expression was graded on the basis of the intensity of staining within the tumor cells. Only nuclear staining was considered positive, while isolated cytoplasmic staining whether in tumor tissue or in few foci of benign prostatic tissue found adjacent to tumor was ignored. The scoring system used for evaluation of Cyclin D1 expression was as follows:
1. Negative (no staining)
2. Mild (nuclear staining of <10% cells),
3. Moderate (staining of 10-49% cells)
4. Strong (staining of ≥50% cells).

**Evaluation of Ki 67 expression:**
The tumors were divided into five groups regarding the percentage of Ki-67 nuclear stained cells. The scoring system used for evaluation of Ki-67 expression was as follows:
1. Cases in which the percentage of stained cells was ≤2% were considered negative.
2. Cases with Ki-67 index of >2% - ≤25% were considered 1+
3. 26-50% as 2+
4. 51–75% as 3+
5. 76–100% as 4+

**Statistical analysis:**
SPSS (statistical package for social sciences) version 22.0 was used for data management and data analysis. Mean ±standard deviation described quantitative variables and median with range when appropriate (distribution did not follow normality). Number and percentages described qualitative data and Chi-square or Fisher exact tested proportion independence. For comparing mean values of 2 independent groups, parametric and non-parametric t test were used. For comparing means of more than two independent groups one way ANOVA (analysis of variance) and Kruskal Wallis ANOVA were used. Correlation analysis was used to show strength and significance of association between quantitative variables. P value is always 2 tailed and significant at 0.05 level.

**Results:**
Among the fifty studied cases, twenty five (50%) were previously diagnosed as prostatic adenocarcinoma, fifteen cases (30%) were diagnosed as BPH and ten (20%) cases were diagnosed as PIN based on hematoxylin and eosin-stained sections.

The age of the studied cases was between 50 -85 years old and the highest median was 67 which was detected in prostatic carcinoma group. The preoperative serum PSA in the studied cases ranged from 1 ng/dl up to 313 ng/dl with a highest PSA mean value seen in prostatic carcinoma group (Table 1).

**Table 1:** Comparison of age and pre-operative PSA level in the studied groups.

| Variable | Prostatic cancer N=25 | BPH N=15 | PIN N=10 |
|----------|-----------------------|----------|----------|
| **Age**  |                       |          |          |
| Minimum  | 55                    | 50       | 50       |
| Maximum  | 83                    | 85       | 80       |
| Mean     | 67                    | 65       | 66.5     |
| **PSA**  |                       |          |          |
| Minimum  | 1                     | 2        | 3        |
| Maximum  | 313                   | 26       | 32       |
| Mean     | 56.43                 | 6.07     | 12.32    |
The Gleason scoring of the studied prostatic carcinoma cases ranged between 4-10. The most prevalent score in the studied cases was Gleason score 8 (28%) (Table 2).

Table (2): Distribution of prostatic carcinoma cases according to Gleason score

| Gleason Score | Number of cases | Percentage (%) |
|---------------|-----------------|----------------|
| Score 4       | 1               | 4%             |
| Score 5       | 1               | 4%             |
| Score 6       | 5               | 20%            |
| Score 7       | 6               | 24%            |
| Score 8       | 7               | 28%            |
| Score 9       | 4               | 16%            |
| Score 10      | 1               | 4%             |
| **Total**     | **25**          | **100%**       |

Regarding Cyclin D1 immunohistochemical staining (Figures 1, 2); there was a statistically significant difference in expression of cyclin D1 between BPH compared with PIN and carcinoma cases (P < 0.001) with the highest expression in carcinoma cases (Table 3).

Table (3): Cyclin D1 expression in all studied cases.

| Cyclin D1 scoring | Prostatic cancer cases N=25(100%) | PIN N=10 (100%) | BPH N=15 (100%) |
|-------------------|-----------------------------------|----------------|-----------------|
| Negative          | 2 (8)                             | 2 (20)         | 12 (80)         |
| Mild              | 5 (20)                            | 4 (40)         | 3 (20)          |
| Moderate          | 10 (40)                           | 2 (20)         | 0 (0)           |
| Strong            | 8 (32)                            | 2 (20)         | 0 (0)           |

Figure (1): Prostatic adenocarcinoma showing strong nuclear Cyclin D1 staining (≥50%). (Cyclin D1 x200)
No significant relationship was found between the intensity of Cyclin D1 expression in prostate cancer cases and other clinicopathological factors as age of the patient (P=0.687) and preoperative PSA (P=0.286). Also there is no significant association or correlation was found between the intensity of Cyclin D1 expression and Gleason scoring of cancer prostate cases (P=0.337) (Table 4).

Table (4):- Correlation between Cyclin D1 expression in prostatic carcinoma cases and age, PSA and Gleason score:

|                | Age | PSA | Gleason score |
|----------------|-----|-----|---------------|
| Cyclin D1      | 0.085 | 0.222 | 0.200 |
| P value        | 0.687 | 0.286 | 0.337 |

Regarding Ki67 immunohistochemical staining (Figures 3, 4); there was a statistically significant difference in expression of Ki-67 between BPH and prostate carcinoma is observed (P value of < 0.001) (Table 5).

Table (5):- Ki-67 expression in all studied groups.

| Ki-67 scoring | Prostatic cancer cases N=25 (100%) | PIN cases N=10 (100%) | BPH cases N=15 (100%) |
|---------------|-------------------------------------|-----------------------|-----------------------|
| Negative(≤2%) | 5 (20)                              | 3 (30)                | 14 (93.3)             |
| +1 (>2% - ≤25%) | 1 (4)                               | 1 (10)                | 1 (6.7)               |
| +2 (26-50%)   | 7 (28)                              | 3 (30)                | 0 (0)                 |
| +3 (51-75%)   | 8 (32)                              | 3 (30)                | 0 (0)                 |
| +4 (76-100%)  | 4 (16)                              | 0 (0)                 | 0 (0)                 |
No significant relationship was found between the intensity of Ki-67 expression in prostate cancer cases and age of the patients (P=0.616), preoperative PSA (P=0.199), but there is statistically significant positive correlation between Ki-67 positivity and increased Gleason’s score (P < 0.001) (Table 6).

Table (6):- Correlation between Ki-67 expression in prostatic carcinoma cases and age, PSA and Gleason score:

| Ki-67 | Pearson Correlation | Age  | PSA  | Gleason score |
|-------|---------------------|------|------|---------------|
|       | 0.105               | 0.266| 0.731|               |
| P value | 0.616              | 0.199|      | < 0.001       |

A statistically significant positive correlation was observed between Cyclin D1 and Ki-67 expression in cancer group, PIN group and BPH group with P value 0.03, 0.001 and 0.04 respectively.
Discussion:
In the present study, twenty three of the prostate cancer cases (92%) revealed positive nuclear staining for Cyclin D1. Nearly similar results were given by previous studies performed by Gupta et al. (2014), Ozbek et al (2000), Ueda et al.(2001) and Anis et al (2013) which reported positive nuclear Cyclin D1 expression ranging between 84.6% and 100%, but the results were in contrast with Kallakury et al.(1997) and Shihaishi et al. (1998) which noticed positive nuclear Cyclin D1 expression in 22%, 30.3% and 11% of their studied cases respectively. Different techniques and methods used for detection of Cyclin D1 in this study might explain the discrepancy in results.

Regarding PIN, eight cases (80%) showed positive Cyclin D1 expression. These results were close to previous studies by Gupta et al. (2014) and Anis et al. (2013) which reported positive nuclear Cyclin D1 expression in all studied PIN cases (100%).

In BPH group, only 3 cases (20%) showed positive nuclear Cyclin D1 expression. These results were in harmony with a previous work performed by Comstock et al. (2007) that reported positive nuclear Cyclin D1 expression in 13% of all cases. On the other hand, other works reported higher percentage of positively stained cases as it was 53.8% in the study done by Ueda et al.(2001) and 72.2% in Gupta et al. (2014).

No significant association or correlation was found between the intensity of Cyclin D1 expression in prostate cancer cases and other clinicopathological factors as age of the patient, preoperative PSA and Gleason scoring of cancer prostate cases. These results go with most of the previous researchers, but they were in contrast with Ozbek et al. (2000) which observed a positive correlation between Gleason grade and staining intensity of Cyclin D1.

There was a statistically significant difference in Cyclin D1 expression among BPH, PIN and carcinoma cases with the highest expression in carcinoma cases. These results were in concordance with a previous work by Gupta et al. (2014) which observed that cyclin D1 expression tends to increase in malignant prostate tissue.

Regarding the expression of Ki-67 in the studied cases, twenty (80%) of the cancer prostate cases showed positive Ki-67 expression. Our results are also close to those observed by other researchers; Muñoz et al (2003) and Verma et al (2015).

In PIN group; seven (70%) cases showed positive Ki-67 staining. These results were different from those in the study done Sasor et al. (2000) in which only 3(30%) out of 10 PIN cases exhibited Ki-67 positivity.

In the present study only one case (6.7%) of BPH cases showed positive Ki-67 expression. These results are close to a previous study by Verma et al (2015) which reported that Ki-67 was expressed in only one out of 10 (10%) of BPH cases.

A statistically significant difference in expression of Ki-67 between BPH and prostate carcinoma was observed and this agrees with results of previous works.

There was no significant association or correlation between the intensity of Ki-67 expression in prostate cancer cases and age of the patients, preoperative PSA, but there is statistically significant positive correlation between Ki-67 positivity and increased Gleason’s score and this is in concordance with other studies which concluded that Ki-67 can be used as a prognostic factor for prostate cancer. But in contrast to a single work performed by Muñoz and his colleagues (2003) which detected no significant difference between the Gleason's score and Ki-67 immunostaining.

In this study a statistically significant positive correlation was observed between Cyclin D1 and Ki-67 expression in cancer group, PIN group and BPH group, and this was similar to a previous study by Aaltomaa and his colleagues (2006).
Conclusion:
This study concluded that in prostatic carcinoma; Cyclin D1 immunohistochemical expression may help in diagnosis but it has no role as a prognostic factor. It was also concluded that Ki-67 can be applied along with other prostate cancer prognostic factors.

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