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RESEARCH

# Expression of Estrogen Receptor β and Ki 67 in Benign & Malignant Human Prostate Lesions by Immunohistochemistry

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**Abstract** Estrogen regulates the growth of prostate through two receptors Estrogen receptor α & β of which ERβ is proposed to be antiproliferative. There is a wide variation in the results of various studies regarding the localisation, level of expression of ERβ in benign & malignant lesions of prostate and its relation to the grade of tumor emphasizing the need for additional studies to standardize the distribution of this receptor in prostate. This was a prospective study conducted in Department of Pathology, UCMS, Delhi, evaluating ERβ & Ki 67 immunoexpression in 60 cases of benign and malignant lesions of prostate (30 each). Tissue for study included prostatic core biopsy and TURP chips. After histomorphological diagnosis, immunohistochemical staining was performed using a monoclonal antibody. Nuclear expression of ERβ & Ki67 was evaluated and compared between the two study groups (benign & malignant lesions) using Pearson chi square test. ERβ was predominantly localized to nuclei of secretory epithelium of prostatic glands. Expression of ERβ was higher in benign glands compared to carcinoma. However, majority of carcinomas retained ERβ expression though at much lower levels. Expression of Ki 67 was higher in carcinoma than benign hyperplasia. There was no correlation between the ERβ status, Ki 67 expression & grade of tumor. Expression of ERβ is downregulated in carcinoma compared to benign hyperplasia and is consistent with its chemopreventive role in prostate. It might have a therapeutic implication as agonists' targeting this receptor could be a part of treatment protocol for those patients of carcinoma who retain this receptor at significant levels.

**Keywords** ER β · Ki 67 · Benign nodular hyperplasia prostate · Carcinoma prostate · Estrogen · Immunohistochemistry

**Abbreviations**

| | |
|---|---|
| *ER α* | Estrogen receptor alpha |
| *ER β* | Estrogen receptor beta |
| *AR* | Androgen receptor |
| *AMACR* | alpha methyl acyl co A racemase |
| *BPH* | benign prostatic hyperplasia |
| *H & E* | Haematoxylin & Eosin |
| *EDTA* | Ethylene diaminetetraacetic acid |
| *HRP* | Horse Radish peroxidase |
| *DAB* | di-amino-benzidinetetrahydrochloride |
| *PSA* | Prostate specific antigen |
| *PBS buffer* | Phosphate buffered saline |

## Introduction

The role of androgens in pathogenesis of prostate carcinoma is well known. Recently studies have revealed the role of estrogen signaling pathways in the carcinogenesis of prostate. The

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function of estrogen in regulation of normal and abnormal growth of prostate is an area of imminent research. Many authors have attempted but left conflicting results.

Several phytoestrogens in the diet bind to estrogen receptors and activate detoxification enzymes such as glutathione-S-transferase in prostatic epithelium highlighting the chemopreventive role of estrogen [1–3]. While few experimental studies in animals have shown that chronic treatment of rats with testosterone leads to high incidence of prostate cancer when combined with estrogens [4–6]. The opposing effects exerted by estrogens on prostatic epithelium are proposed to be mediated by two types of receptors: ER α and ER β [7, 8].

Studies performed on benign human prostatic tissue have shown that ER α expression is limited to prostatic stromal cells and the basal cell layer, while secretory luminal cell types of the prostatic epithelium lack ER α at the mRNA and protein level [9, 10]. Considering expression of ER β , its localization in human prostate tissue is not well recognized. There are conflicting results by different authors on its location in prostatic tissue. According to some authors ER β is highly expressed in rat prostate epithelial cells and in the secretory epithelium of normal human prostate, where the levels of ER β mRNA are higher than the levels of ER α mRNA [11, 12]. A significant study by Leav et al. immunolocalized ER β predominantly in basal cells using a polyclonal antibody. They showed that ER α was present in stromal cells and AR was predominantly localized in the nuclei of differentiated secretory cells [13]. In contrast to the above result a major study by Fixemer etal who used a monoclonal antibody, detected ER β predominantly in secretory luminal cells of glands and to a lesser extent in the basal cells [14].

Animal Studies have shown that ER α stimulates cell proliferation in the immature rat uterus, while the ER β restrains ER α activation [15]. It has been suggested that ER β, acting through estrogens, may protect the normal prostate epithelium from oxidative injuries, uncontrolled cell proliferation and neoplastic transformation by activating chemoprotective detoxification enzymes [13, 16].

Referring to ER α in prostatic carcinoma, Bonkhoff et al. reported a high expression of this receptor in metastatic and androgen insensitive lesions [10]. Regarding expression of ER β, different authors have given variable results in primary prostatic carcinoma, metastatic lesions and in tumors which become refractory to androgen ablation therapy. The observation that older ER β -null mice develop prostatic hyperplasia have led to the hypothesis that loss of ER β may be a mechanism by which prostate epithelial cells escape normal control of proliferation and lower levels of ER β can contribute to prostatic neoplasia [17, 18]. Study by Leave et al. showed loss of ER β expression, at both the transcriptional and translational levels during prostatic carcinogenesis and tumor progression. This may signify the loss of an important role the receptor would normally play in inhibiting growth of the prostate that could contribute to neoplastic development [13]. In contrast to the above studies a major study by Fixemer etal identified ER β in nearly all-primary prostatic adenocarcinoma [14].

As there is wide variability in the results of different studies regarding expression of ER β in prostate carcinoma and its relation to the grade of tumor, there is a need for additional studies to standardise the distribution of this receptor in human prostatic tissue. This will help in understanding its role in the regulation of prostate epithelial cell proliferation at different stages in the development of carcinoma prostate.

As this receptor is proposed to be anti proliferative a better understanding of the function of ER β in the evolution of prostate carcinoma could strongly impact on the therapeutic options for patients who have ER β expressing tumors.

We conducted this study to find out the pattern of expression of ER β in prostatic carcinoma and nodular hyperplasia by immunohistochemistry and compare the results in the two groups. We also determined the proliferation index by Ki 67 immunoexpression in benign and malignant prostate lesions. As ER β is proposed to be antiproliferative and Ki 67 a marker of proliferation, we tried to find out the correlation between ER β and Ki 67 expression and ER β with the grade of the tumor, if any.

## Materials and Methods

It was a prospective study evaluating ER β & Ki 67 expression in benign and malignant prostate lesions. Cases diagnosed as benign hyperplasia prostate or carcinoma prostate were taken from surgery department, UCMS & GTB Hospital during the time period January 2010 to January 2012. Clinical details, PSA levels and findings on imaging if any, were recorded. 30 cases each of carcinoma prostate and nodular hyperplasia prostate/benign prostatic tissue confirmed on histopathological examination were included in the study. Tissue for study included prostatic needle biopsy and chips of transurethral resection of prostate. All cases where tissue obtained was scant for immunostaining were excluded from the study.

The tissue was preserved in 10 % buffered formalin and processed routinely. Five 4 micron-thick sections were prepared from each tissue block. One section was stained with Haematoxylin and Eosin (H & E) for morphologic diagnosis and Gleason's score. Gleason score was graded as: 2–4 (grade 1), 5–6 (grade2), 7 (grade 3), 8–10 (grade4) [19]. The rest of the sections were mounted on poly L lysine coated slides. 1 section was evaluated for immunoexpression of 34 β E12 and another section for alpha methylacyl Coa Racemase (AMACR). The other 2 sections were subjected to ER β and Ki 67 immunohistochemical staining.

*Immunohistochemical Staining* After deparaffinization in xylene, the sections were hydrated through a series of graded alcohols and distilled water. Heat induced epitope retrieval was initially performed on control tissue by heating sections in citrate buffer (pH 6.2) for 15 min at 98 °C in a microwave retrieval system. It failed to give any positive immunohistochemical results. Antigen retrieval was then done in Tris-EDTA buffer at pH 9.0 which yielded good results. After antigen retrieval sections were incubated in 3 % $H_2O_2$ for 10 min to inhibit the endogenous peroxidase activity. The slides were washed thrice with Tris buffer at pH 7.6. Mouse monoclonal antibody to ER β directed against synthetic peptide derived from the C terminus of the human Estrogen receptor β 1 isoform (Acris antibody; Clone- PPG5/10) was used at a dilution of 1:10 diluted in Tris buffer. Slides were incubated with diluted monoclonal antibody in humid conditions at 4 ° C overnight. The slides were then washed thrice with Tris buffer (pH 7.6) for 5 min each. The secondary biotinylated antibody was applied for 30 min at room temperature. The slides were then washed with Tris buffer thrice for 5 min each. This was followed by application of peroxidase labelled antibody (Streptavidin- HRP labelled; Biocare) for 30 min at room temperature. Slides were again washed thrice with Tris buffer thrice for 5 min each. DAB was applied (3, 3′-di-amino-benzidinetetrahydrochloride) as chromogen for 5 min. Slides were then rinsed in distilled water for 5 min. Counterstaining was done with Harris modified haematoxylin. Sections were dehydrated in ascending grades of alcohol and cleared in xylene for three changes and cover slips were applied. We also stained a section of normal prostate to determine the expression of ER β. With every batch of test slides a positive control of ovary with granulosa cells for ER β was stained. Primary antibodies replaced by buffer were used as a negative control for both stains.

*Evaluation of Estrogen Receptor β Expression* Nuclear staining within the cell, whether weak or strong, was considered positive. Given previous evidence that ER *β* is a steroid receptor localised to the nucleus, only positive nuclear immunostaining was scored [14]. 400 epithelial cells (secretory) were counted in each case to determine the percentage of immunostained nuclei across all the cancer areas present. ER *β* scores were expressed as the percentage of cells demonstrating nuclear immunoreactivity and was scored as follows: <10 % (1+), 11–40 % (2+), 41–60 % (3+), 61–80 %( 4+), >80 %( 5+).

*Immunohistochemical Staining for Ki 67* Similar procedure was followed for evaluating proliferative index by Ki 67 immunostaining. Antibody used for Ki 67: rabbit monoclonal prediluted antibody, Clone: SP 6 (Cell Marque, California, USA). A section of lymph node was also stained as a positive control. The percentage of immunostained nuclei across the cancer areas was calculated and grade as follows: <1 % (1+), 1–5 % (2+), ≥5–10 %( 3+), ≥10–20 %( 4+), ≥20 % (5+) [20].

Statistical Analysis

Immunoexpression of the study variables ER β and Ki 67 was compared between two groups- group 1 (malignant) & group 2 (benign), using Pearson chi square test and Fischer exact test. $p$ value<0.001 was considered significant. Pearson correlation was used to find out correlation between ER β and Ki 67-IHC score in both the study groups, and the correlation of ER β with Gleason score if any.

## Results

The age of patients with carcinoma prostate varied from 45 to 80 years with a median age of 70 years. Patients with carcinoma prostate had elevated serum PSA levels ranging from 10 to 649 ng/ml with all patients having value more than 10 ng/ml. On histopathologic examination all cases in the malignant group were adenocarcinomas. Majority were high grade tumors (50 %) with a Gleason score of 8–10. ER β immunoexpression was seen in 100 % of cells in the secretory epithelium of normal prostate. Few nuclei in the basal cells and stromal cells also showed positive nuclear expression. Cytoplasmic staining was seen in all cases in epithelial compartment but was considered non specific. Therefore, ER β was seen predominantly in the secretory epithelial compartment compared to basal cell layer and the stromal cells.

Cases of benign hyperplasia showed a high expression of ER β in the secretory epithelium. Majority (83.3 %) expressed ER β at high levels (score4+, 5+) as shown in Fig. 1.

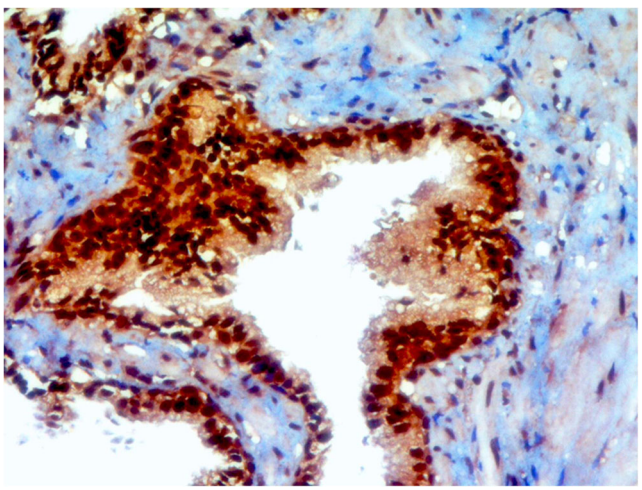

**Fig. 1** High power view showing strong nuclear immunoexpression of ER β in secretory epithelial cells (400X)

Cases of carcinoma prostate showed relatively reduced expression of ER β. One third of patients expressed ER β at low levels with a score of 1+,2+ while all patients (30/30) with BPH expressed ER β at high levels with score more than 3+ (Figs. 2 and 3).

A smaller number (9/30) of patients with carcinoma prostate expressed ER β (score 5+) at high levels compared to a large number (22/30) in the benign group who showed high nuclear expression of ER β (Fig. 2). ER β expression was compared in two groups by Pearson Chi Square and Fisher's exact test and the result was statistically significant ($p$ value<0.001).

Ki67 expression was low (<5 %) in most of the benign cases (86.3 %) as shown in Fig. 4. On the contrary proliferative index was higher in carcinoma prostate. A large number of cases (46 %) in this group had values >10 % (score 4+, 5+) (Figs. 4 and 5). Proliferation index was as high as 75 % in one case. Pearson Chi Square and Fisher's exact test was used to analyze the above data and $p$ value was significant (<0.001).

We also correlated Ki 67 with ER β in the two study groups (carcinoma and BPH) using the Pearson correlation but the results were found to be insignificant ($p$ value>0.001). No correlation was found between the Gleason score and levels of ER β expression.

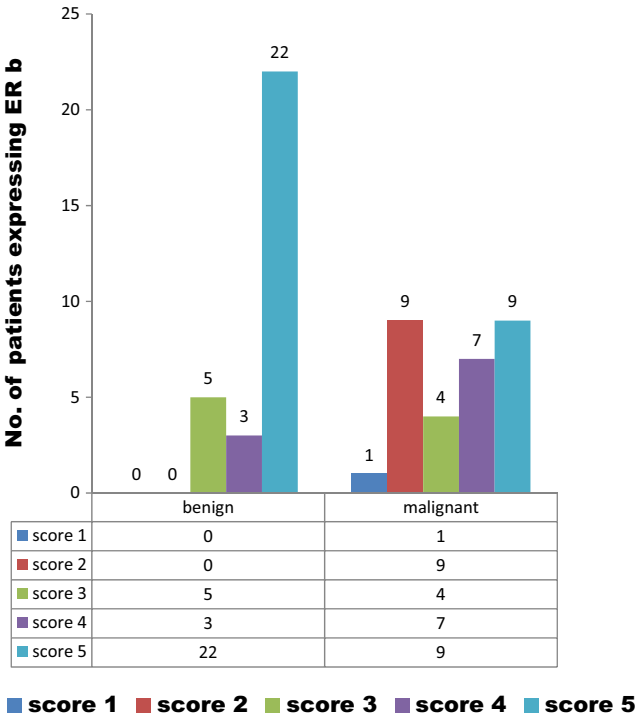


**Fig. 2** Chart showing comparative ER β nuclear expression (score1-5) in carcinoma and benign hyperplasia prostate (y axis representing no. of patients). Expression is reduced in carcinoma but retained in most patients though at lower level

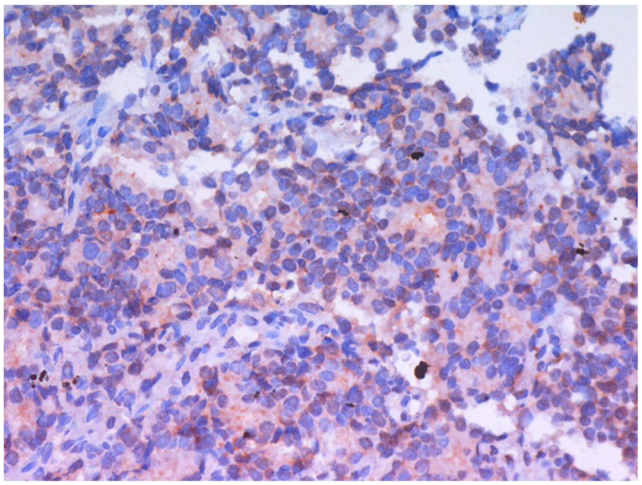

**Fig. 3** Photomicrograph of carcinoma prostate showing loss of ER β expression on immunohistochemistry (400X)

## Discussion

Conventional adenocarcinoma is the most common type of epithelial malignancy of prostate as was seen in the present study [21]. It has been demonstrated and supported by few studies that ER β is reduced in carcinoma prostate compared to nodular hyperplasia as it is anti proliferative [13, 22]. If the hypothesis that ER β is lost during carcinogenesis is true, it will help in guiding therapy in prostate cancer prevention trials. The results of different studies on localisation and expression of ER β in carcinoma prostate are highly variable.

Our results are in concordance with study by Fixemer et al. and Horvath et al. who immunolocalised the receptor predominantly in secretory luminal cell types and to a lesser extent in

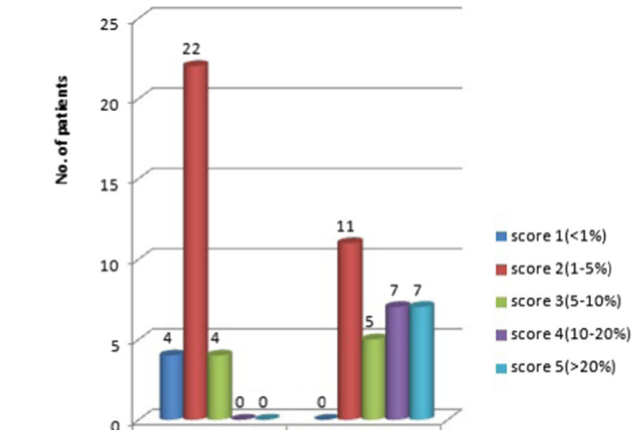


**Fig. 4** Chart showing Ki 67 expression (score1-5) in nodular hyperplasia prostate and carcinoma (y axis representing no.of patients). Expression is higher in carcinoma

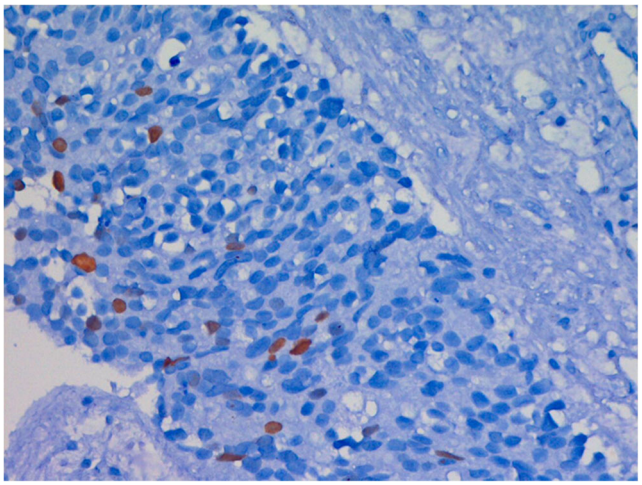

**Fig. 5** Photomicrograph showing higher Ki 67 expression in carcinoma prostate (400X)

basal cells [14]. Also studies in rat and murine prostate indicate that ER β is highly expressed in the luminal cells [11, 18]. In contrast to this finding, a major study by Leav et al. suggested that this receptor is predominantly present in basal cells of normal glands and to some extent in stromal nuclei with occasional positive nuclear membrane staining in secretory cells [13].

The variable results on the localization of the ER β in benign human tissue may be due to different specificity of the primary antibodies used in above studies. In particular, the GC-17 antibody used by Leav et al. was a polyclonal antibody prepared against the F domain of ER β. It identifies a post-transcriptionally modified form of the long-form ER β while the monoclonal antibody used in our study recognizes the long and the short form of the ER β directed against synthetic peptide derived from the C terminus of the human Estrogen receptor β 1 isoform [13].

We evaluated the levels of immunoexpression of ER β in benign prostatic hyperplasia and carcinoma prostate and compared them. The nuclear expression of ER β in malignancy varied from 9 to 100 and 51–100 % in BPH. All cases of carcinoma prostate in the present study show a positive ER β expression (nuclear expression >9 %) though at much lower levels (Fig. 2). Our results corroborate with study by Fixemer et al. where all primary adenocarcinomas retained positive ER β expression [14]. Similar results have been given in another recent study by Asgari M where majority of (92.1 %) cases of carcinoma prostate showed positive ER β expression [24].

Gabal SM et al. also demonstrated diminished ER β levels in carcinoma compared to benign hyperplasia like our study, but in contrast to our study, levels of ER β expression were markedly reduced in their study [22]. Only 17.2 % of prostatic adenocarcinomas showed positive ER β (>5 % nuclear positivity) whereas all cases of carcinoma (100 %) in our study show nuclear expression >5 %. Thus levels of nuclear expression of ER β were not as low as documented in their study. Similarly Horvath et al. also reported progressive loss of ER β in prostatic hyperplasia and to a greater extent in invasive cancer [23]. Only 11 % of carcinoma patients in their study expressed ER β at levels >5 %.

Contrary to the above findings Fixemer et al. concluded that ER β levels are retained in all primary adenocarcinomas and metastatic carcinomas (similar to nodular hyperplasia) at high levels but reduced significantly in recurrent carcinoma [14]. Eighty-seven percent of primary tumors in their study retained the high-level expression of the ER β whereas only 13 % revealed lower rates. The data from Leav et al. also show markedly decreased levels of ER β at protein and transcriptional levels in high grade dysplasia & in Gleason grade 4/5 tumors but most Gleason grade three tumors and tumors metastatic to bone and lymph nodes retained high expression of ER β as in benign prostate. The authors have not clearly explained the reappearance of ER β in grade three tumors [13]. So our study indicates that majority of prostate adenocarcinomas retain positive ER β expression though at significantly lower levels when compared to benign hyperplasia. There is no obvious explanation for these controversies between different reported results on the levels of ER β expression. It is well known that imperfect antibody specificity or different primary antibodies, ineffective antigen retrieval and tissue-processing methods, or the presence of unknown isoforms of ER protein may affect immunohistochemistry performance.

A possible implication of ER β in neoplastic growth control is supported by the findings of a selective loss of ER β protein in colon adenocarcinoma and ovarian cancer [25, 26]. A recent experimental study on human prostate cancer cell lines suggested that ER β acts as a tumor-suppressor by its anti-proliferative, anti-invasive and pro-apoptotic properties [27].

Role of ER β in prostate carcinogenesis has been emphasized through cancer prevention trials also. In one of the Prostate Cancer Prevention Trials more than 18,000 healthy volunteers were randomly assigned to receive either finasteride or placebo. The incidence of tumors with a high Gleason grade was more in the finasteride group than in the placebo group. Finasteride is a drug that acts by suppressing ER β and preventing the differentiation of epithelium. This mechanism could account for the higher incidence of poorly differentiated tumors in the finasteride group. Above authors suggested that finasteride be combined with an ER β agonist in future studies of chemoprevention of prostate cancer [28]. Our study suggests the loss of ER β during carcinogenesis and as proposed by the above cancer prevention trial it may have a therapeutic implication in chemoprevention of prostate cancer. Studies suggest that in a normal prostate gland the basal cell layer is responsible for cell proliferation and has lower levels of ER β as indicated by our study. Conversely, secretory luminal cells with high ER β levels constitute the differentiation compartment, supporting its antiproliferative role [29, 30].

There was no correlation between Gleason score and expression of ER β. Our results are in concordance with study by Fixemer et al. who reported similar findings [14]. Leave et al. reported loss of ER β expression in high-grade dysplasia, its reappearance in grade three cancers, and its diminution in grade 4/5 neoplasms [13].

A possible explanation of above discrepancies in levels of ER β expression and relation to the grade of tumor could be because of specificity of the antibody, different dilutions of antibodies used and unknown isoforms of ER β which may react with the antibody. We used a specific mouse monoclonal antibody to ER β isoform 1(clone PPG 5/10) at a dilution of 1:10 (Acris antibody) directed at C region of ER β. This antibody does not cross react with ER a receptor. The same antibody was used by Fixemer et al. at a dilution of 1:50 in PBS [14] while Leave et al. used a novel antibody directed against the F domain of ER β, a region that has no homology with the ER α receptor [13]. Other than specificity of the primary antibody, tissue fixation and processing might affect ER β expression in the tissue by IHC.

Considering Ki 67, expression was higher in carcinoma prostate compared to benign hyperplasia. Majority of cases with carcinoma (46 %) had proliferation index >10 % (Fig. 4) while most cases of BPH (73.3 %) had values between 1 and 5 %. Our results are in concordance with other studies on immunoexpression of Ki 67 in prostatic neoplasia [31, 32]. However we did not find any correlation between immunoexpression of Ki 67 and ER β.

## Conclusion

We conclude that there is reduced ER β expression in adenocarcinoma prostate when compared to benign hyperplasia but proportion of carcinoma cases showing positive ER β expression is much higher in comparison to other studies by various authors [22, 23]. Therefore, most carcinomas of prostate retain ER β immunoexpression but at much lower levels than benign prostatic hyperplasia.

There is great need to standardize the immunoexpression of this receptor using highly specific primary antibodies at different stages of neoplasia in order to conclude on its role in pathogenesis of carcinoma prostate. It might have an implication on treatment of carcinoma prostate as therapy targeting this receptor could be a part of treatment protocol for those few patients of carcinoma who retain this receptor at significant levels. As there is loss of this receptor from benign hyperplasia to carcinoma, due to its anti-proliferative role an agonist to ER β can be a part of prostate cancer prevention trials in normal subjects.

**Conflicts of Interest** None.

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