ORIGINAL ARTICLE

Ki-67 and PCNA expression in prostate cancer and benign prostatic hyperplasia

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Abstract

Objective: Ki-67 is a proliferation-associated nuclear antigen and is expressed in all cycling cells except for resting cells in the G0-phase. PCNA is an acidic nuclear protein and has been recognized as a histologic marker for the G1/S phase in the cell cycle. Ki-67 and PCNA labeling indices are considered to reflect cell proliferation, particularly, growth fraction. The purpose of this study is to investigate the expression levels of Ki-67 and PCNA in prostate cancer (PCa) and benign prostatic hyperplasia (BPH) and their potential on the early diagnosis of PCa.

Methods: Human prostate cancer cell lines LNCaP and PC-3, human normal prostate epithelial cell line HuPEC, tissues from patients with PCa (121 cases) and BPH (45) and 36 normal cases were examined for the expression of Ki-67 and PCNA by Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Then, the association of Ki-67 and PCNA expression with clinical grading of PCa was analyzed by immunohistochemistry staining.

Results: The ratios of PCNA and Ki-67 expression levels in LNCaP and PC-3 were higher ($P < 0.05$, $P < 0.001$) than that in HuPEC. The two markers were differentially expressed in three tissues and showed increased expression in PCa ($P < 0.05$) and BPH ($P < 0.05$), relative to human normal prostate tissues. Compared with BPH, the ratio of Ki-67 and PCNA expressed in tumour tissue was increased ($P < 0.05$). The increase of Ki-67 was greater than that of PCNA. Expression of the two markers increased after different grading of PCa cases. The values of Ki-67/PCNA

were: 0.073 in grade I PCa tissues, 0.119 in grade IIa PCa tissues, 0.141 in grade IIa PCa tissues, 0.234 in grade III PCa tissues.

Conclusion: The combination of Ki-67 and PCNA, specific proliferative markers of PCa, may improve the accuracy of early diagnosis of prostatic cancer.

Prostate cancer (PCa) is the second most frequent cause of male cancer-related death in the United States of America (USA) and Western Europe. Its incidence is continuously rising, with over 200 000 new cancers and 35 000~40 000 deaths per year.¹ Carcinogenesis and the mechanisms influencing the progression and prognosis of PCa is a multistep process, involving both genetic insults to epithelial cells and changes in epithelial-stromal interactions.² Despite extensive research PCa is not understood.

There are two proliferative markers—Ki-67 and proliferating cell nuclear antigen (PCNA). Ki-67 antigen is expressed in proliferative cells throughout the G1, S, G2, and M phases, and provides a reliable index of cellular proliferation.^{3,4} PCNA is an acidic nuclear protein, expressed mainly in phase S of the cellular cycle. It becomes active, in various tissues par-

ticularly in nervous tissue, as a first response to multiple insults.^{5,6}

Recent studies have demonstrated that identification of proliferative activity in a tumour may predict the biological behaviour.^{7,8} There are no reports of the relation between proliferating activity and clinical aspects of PCa. Therefore, to investigate the potential of Ki-67 and PCNA on the early diagnosis of PCa, we observed the expression of these markers in PCa and benign prostatic hyperplasia (BPH).

Methods

Human prostate carcinoma cell line LNCaP, hormone dependent (ATCC # CRL-1740), human prostate carcinoma cell line PC-3, hormone independent (ATCC # CRL-1435), and human normal prostate epithelial cell line HuPEC (ATCC # CRL-1059) were used. Cell lines were maintained in Opti-MEMR I medium supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine at 37 °C in 5% CO₂.

Approval was obtained from the local research and ethics committee for use of tissue. Specimens were obtained from patients (121 with PCa, 45 with BPH and 36 normal cases) undergoing transurethral resection of the prostate (TURP) or transvesical prostatectomy (TP) for BPH, or TURP or radical prostatectomy (RP) as treatment for PCa at the Urinary Surgery Department of First Municipal People's Hospital, Guangzhou Medical College, Guangdong, P.R.China and The Fourth PLA Medical University, Xi'an, P.R.China. None of the patients had undergone chemotherapy or radiotherapy before surgery. The age of the patients, maximum tumour size, depth of wall invasion and histological grade were obtained from histopathology reports. Staging of PCa was done according to the Tumour-Node-Metastasis Stage Grouping.⁹ MTT, DMSO, and DNase I were purchased from Sigma. Opti-MEMR I reduced-serum medium and Trizol were from Gibco-BRL Life Technologies. Plasmid Purification Kit was from Qiagen. All chemicals, reagents and solvents were of the high-

est grade available and were used as directed by the manufacturers. Distilled water used was obtained in our laboratory by an ion-exchange and distillation process.

RNA isolation

Total RNA was extracted from the LNCaP, PC-3 and HuPEC pellets, and the human tissues with a phenol/guanidine isothiocyanate-based reagent (Trizol™, Gibco-BRL, Scotland.). Briefly, 1 ml Trizol and 200 µl chloroform were added to each tube. The RNA was then precipitated with 500 µl isopropanol (v/v) and washed with 75% ethanol and air-dried. The purified RNA was then dissolved in 10 µl diethyl pyrocarbonate (DEPC)-treated water, optical density measured at 260/280 wavelength and stored at -80°C prior to use in the synthesis of cDNA.

Fluorimeter-based RT-PCR

RNA was reverse transcribed using the Superscript™ Preamplification System (Life Technology). 500ng total RNA were mixed with 500ng oligo (dT) primers and incubated at 70°C for 10 min. The mixture was then chilled on ice and incubated with a 1× reverse transcriptase buffer (50 mM Tris-HCl, pH 8.4, 75mM KCl), 3 mM MgCl₂, 500µM of each deoxynucleotide, 10mM dithiothreitol and 200U Superscript II RT reverse transcriptase at 42° for 50 min. The 20µl reactions were further incubated at 70° for 15 min and 2 U RNase H were added to each tube. Following final incubation at 37° for 20 min, the cDNAs were stored at -80° until used.

PCR was performed in ABI 7900HT ((Idaho Technology, Idaho Falls, ID, USA) using SYBR Green I (Biogene) as fluorescein. The following pairs of primers were used: β-actin (161bp): 5'-ACT GGA ACG GTG AAG GTG ACA G-3' (sense), 5'-GGT GGC TTT TAG GAT GGC AAG-3'(antisense); Ki-67 (240bp): 5'-CTG GGT TAC CTG GTC TTA GTT C-3' (sense), 5'- GAG GCT GTT CCT TGA TGA TTT -3' (antisense); PCNA (203bp) 5'- TTT TCT GTC

ACC AAA TTT GTA CCT C -3' (sense); 5'- CTG CAT TTA GAG TCA AGA CCC TTT -3 (antisense). All primers were synthesized by Sangon Co. (Shanghai, China). The PCR profile consisted of an initial melting step of 1 min at 94°, followed by 38 cycles of 15 s at 94°, 15 s at 56 ° and 45 s at 72°, and a final elongation step of 10 min at 72°.

Quantitative determination of Ki-67 and PCNA mRNA

Sample quantification was carried out with Sequence Detection System (SDS2.0) software and constructing a standard calibration curve using serial dilutions of plasmid β -actin DNA of known concentration, from which the concentration of an unknown sample could be determined. To assay inter-assay variation, a least square curve fitting test was used to construct a fit, and the standard error of the data points to the curve was estimated by plotting the log of the sample concentration against the number of cycles the PCR machine undertakes to yield a set of fluorescent data. The standard error was estimated to be 15% on the log and was within the values described for β -actin quantification using competitive PCR.

Immunohistochemistry Staining and Assessment

For immunohistochemistry, tissues were fixed in 10% buffered formalin and embedded in paraffin. Commercially available monoclonal antibodies to PCNA and Ki-67 were used. Immunohistochemical staining was carried out on TMA sections using the avidin-biotin method and a commercially available kit (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA). One paraffin-embedded block of tumour tissue was selected from each case and cut into 4 μ m sections. Deparaffinized sections were treated with methanol containing 3% hydrogen peroxide for 10 min before antigen retrieval using a microwave oven at 95°C for 5 min and cooling at 25°C for 2 hr. After washing with phosphate-buffered saline, blocking serum was applied for 10 min. The sections were incubated with an anti-PCNA monoclonal anti-

body and an anti-Ki-67 monoclonal antibody overnight at 4°C. After washing in phosphate-buffered saline, a biotin-marked secondary antibody was applied for 10 min followed by a peroxidase-marked streptavidin for an additional 10 min. The reaction was visualized using 3, 3'-diaminobenzidine tetrahydrochloride. The nuclei were counterstained with hematoxylin. Positive and negative immunohistochemistry controls were routinely used. Reproducibility of staining was confirmed by re-immunostaining via the same method in multiple, randomly selected specimens.

To determine the expression of PCNA and Ki-67, two experienced pathologists independently examined staining while blind to the clinicopathologic data and clinical outcomes of the patients. The number of positive cells that showed immunoreactivity on the cell nucleus in the representative ten microscopic fields was counted and the percentage of positive cells was calculated.

Statistical analysis

The labeling indices of PCNA and Ki-67 were counted. The mean and the standard deviation (SD) were calculated. SPSS12.0 software was used for all statistical data analyzed by the unpaired t-test. The selected level of significance was $P < 0.05$.

Results

RNA isolation

The purity of RNA extracted from the LNCaP, PC-3 and HuPEC pellets, and human tissues of PCa, BPH and normal cases was very high according to the ratio value of OD260/OD280, which was ranged from 1.9 to 2.0. Therefore, this RNA could be used for following RT-PCR test.

Quantitative determination of Ki-67 and PCNA mRNA

Standard curves of β -actin, Ki-67 and PCNA were produced by SDS2.0 software (Fig.1).

FIG 1 NEAR HERE

TABLE 1. The expression level of Ki-67 and PCNA in LNCaP, PC-3 and HuPEC cell lines

Cell lines	β -actin quantitative result	β -actin relative magnitude	PCNA quantitative result	Relative expression level	PCNA relative magnitude	Ki-67 quantitative result	Relative expression level	Ki-67 relative magnitude	Ki/PCNA
LNCaP	1561291 $\pm$ 16.59	1.782	124239 $\pm$ 22.35	270551	392.552	22691.3 $\pm$ 18.19	49414	15132.602	0.183
PC-3	7895837 $\pm$ 11.23	9.011	94855.9 $\pm$ 13.59	40845.8	59.265	977462 $\pm$ 27.46	420898	128896.307	10.347
HuPEC	876215 $\pm$ 20.57	1.000	689.21 $\pm$ 8.51	689.21	1.000	3.2654 $\pm$ 11.58	3.2654	1.000	0.005

TABLE 2. Comparative expression levels of Ki-67, PCNA and the association of them with the clinical stages of PCa tissues

Cases (n)	β -actin quantitative result	β -actin relative magnitude	PCNA quantitative result	Relative expression level	PCNA relative magnitude	Ki-67 quantitative result	Relative expression level	Ki-67 relative magnitude	Ki-67/PCNA
PCa I (25)	2992568 $\pm$ 9.265	1.341	6918.279 $\pm$ 10.38	6918.279	2.002	508.209 $\pm$ 11.231	508.209	25.094	0.073
PCa IIa (30)	3129863 $\pm$ 1.268	1.403	7207.037 $\pm$ 9.867	7207.037	2.085	856.186 $\pm$ 6.892	856.186	42.281	0.119
PCa IIb (38)	3695421 $\pm$ 8.298	1.656	7825.193 $\pm$ 11.217	7825.193	2.264	1102.032 $\pm$ 10.278	1102.032	54.416	0.141
PCa III (28)	3208564 $\pm$ 12.035	1.438	8127.296 $\pm$ 8.978	8127.296	2.352	1899.026 $\pm$ 11.207	1899.026	93.770	0.234
BPH (45)	2986575 $\pm$ 21.151	1.337	6826.091 $\pm$ 12.362	6826.091	1.980	56.138 $\pm$ 12.292	56.138	2.771	0.008
Normal (36)	2231069 $\pm$ 11.016	1.000	3456.033 $\pm$ 11.673	3456.030	1.000	20.252 $\pm$ 6.181	20.252	1.000	0.006

TABLE 3. Ki-67 and PCNA Positive rates in different human tissues and the association of them with the clinical stages of PCa tissues

Factor	Cases no.	Ki-67 expression		P	PCNA expression		P
		Negative (%)	Positive (%)		Negative (%)	Positive (%)	
PCa tissues							
Depth of wall invasion ^a							
T1	20	65.00	35.00	0.031	75.68	24.32	0.040
T2	38	47.37	52.63		61.76	38.24	
T3	26	32.35	67.65		55.26	44.74	
T4	17	28.38	71.62		49.15	50.85	
TNM stages a							
II+IIa	55	40.58	59.42	0.005	69.49	30.51	0.009
IIb+III	66	21.96	78.04		53.55	46.45	
BPH tissues	45	42.19	57.81	0.026 *	58.26	41.74	0.031 *
Normal control	36	76.82	23.18	0.009 *	82.17	17.83	0.011 *

a Tumor staging of PCa was done according to be Tumor-Node-Metastasis Stage Grouping [9]

* refers to comparison of positive rates of Ki-67 and PCNA in BPH and normal tissues with PCa tissues

FIGURE 1. Serial dilutions of β -actin plasmid (a), Ki-67 (b) and PCNA (c) DNA fragments were amplified in the presence of the dsDNA-specific dye SYBR Green I in order to permit the construction of a standard calibration curve. The regression equation of β -actin standard calibration curve is $Y = -2.867X + 36.54$ and $R^2 = 0.99$ (a), the regression equation of Ki-67 standard calibration curve is $Y = -3.358X + 39.77$ and $R^2 = 0.994$ (b), the regression equation of PCNA standard calibration curve is $Y = -3.03X + 39.61$ and $R^2 = 0.994$ (c).

FIGURE 2. Immunohistochemical staining for Ki-67 in different tissues (Original magnification $\times 200$). A, Ki-67 staining is negative in normal prostate tissue; B, Ki-67 expression was found in cell nucleus in PCa tissues; C, decreasing Ki-67 expression was found in cell nucleus in BPH, and the arrow indicates the positive area in the tissues.

FIGURE 3. Immunohistochemical staining for PCNA in different tissues (Original magnification $\times 200$). A, PCNA staining is negative in normal prostate tissue; B, PCNA expression was found in cell nucleus in PCa tissues; C, decreasing PCNA expression was found in cell nucleus in BPH, and the arrow indicates the positive area in the tissues.

Threshold cycle (Ct) was defined as the cycle number at which the fluorescence signal passed the noise band. Using the standard curve, amplification of input cDNA templates of Ki-67 and PCNA in the ABI 7900HT was quantified. Curve correlation was good, $R^2 > 0.99$.

The quantitative results of Ki-67 and PCNA mRNA expressed in LNCaP, PC-3, HuPEC cell lines and in the human tissues from patients with PCa, BPH and normal cases are shown in Table 1 and Table 2.

The ratios of PCNA and Ki-67 expression in the PCa cell lines were higher ($P=0.05$, $P<0.01$) than in the normal human prostate cell line. Moreover, the two markers were differentially expressed in three kinds of tissues and the increased expression appeared in PCa ($P<0.05$) and BPH ($P<0.05$) tissues, relative to

human normal prostate tissues. Compared with BPH, the ratio of Ki-67 and PCNA expressed in tumour was more than that in BPH ($P<0.05$), but the increasing extent of Ki-67 was larger than that of PCNA, which was the same as changes in PCa cell lines *in vitro*.

Association of Ki-67 and PCNA expression with the clinical stages of PCa is also shown in Table 2. Expression of the two markers increased following different PCa grading. Values of Ki-67/PCNA were: 0.073 in grade I, 0.119 in grade IIa, 0.141 in grade IIa, and 0.234 in grade III PCa tissues.

Immunohistochemical detection of Ki-67 and PCNA

The expression and localization of Ki-67 and PCNA in PCa, BPH and normal prostate tissues were exam-

ined immunohistochemically. Staining varied in the intensity and percentage of positive cells (Figure 1 and 2).

Ki-67 and PCNA were expressed in 67.76% and 64.46 % of PCa cells at various levels and were mainly localized in cell nuclei. The percentages of positive cells in BPH and normal prostate tissues were significantly lower than that in PCa tissues (Table 3).

Association between Ki-67 and PCNA expression and clinical grading of PCa

There was an association between expression of Ki-67 and PCNA and the clinical grading of PCa (Table 3). The incidence of positive cells of Ki-67 and PCNA was more frequent in carcinomas in stages IIb~ III than stage I~IIa ($P=0.005$, $P<0.01$). Positive expression of Ki-67 and PCNA also tended to be associated with deep invasion of tumour cells in capsula prostatica ($P=0.031$, $P=0.040$).

Discussion

PCa is one of the most serious of carcinomas. Resistance to hormone therapy is related to a considerable proportion of PCa deaths with few therapeutic options available thereafter.^{10,11} Recently, molecular tests for cancer cell-associated genes and other genetic markers, being direct indicators of the presence of the cancer cells in the biological specimen, have been predicted to provide improvement in diagnostic specificity^{12,13} As the growth rate of tumour tissue is determined by proliferative activity and cell death, expression of two proliferative markers-- Ki-67 and PCNA were examined in this study to enhance the facility and accuracy of early diagnosis of PCa.

Ki-67 and PCNA labeling indices showed different results. First, they were expressed more in PCa cell lines (LNCaP and PC-3) than in normal prostate epithelial cells. Moreover, up-regulation of Ki-67 in the PC3 cell line was different from that in the LNCaP cell line, which was more distinctive than the latter. This may be due to the proliferation rate of

PC3 cell line (passage every day in our lab) is faster than LNCaP cell line (passage every 6 or 7 days in our lab). Second, increased expression of the two markers was demonstrated in PCa and BPH tissues. Third, compared with BPH, increased expression of Ki-67 and PCNA was shown in PCa tissues. The increase of Ki-67 was larger than that of PCNA, which was the same as changes in PCa cell lines *in vitro*. This suggests that expression of Ki-67 was more sensitive in reflecting the rate of cell proliferation in PCa tissues, which is similar to previous reports.^{14,15}

The Ki-67 antigen is useful for identifying proliferative cells. Although flow cytometry methods showed that Ki-67 is expressed in all cell cycle phases except G0 phase, its detection by immunohistochemical methods is limited to its appearance in the late G1 phase with maximum expression in prophase and metaphases.¹⁶ Immunoexpression then diminishes in anaphase and telophase, levels being undetectable during most of the interphase. Thus, Ki-67 is considered as a mitotic activity indicator. An increase in Ki-67 expression indicates a rise of the mitotic activity and of the cell proliferation. Moreover, PCNA is a 36-kd DNA polymerase delta auxiliary protein that complexes with cyclin D and cyclin-dependent kinases. It is involved in the proliferation of neoplastic as well as non-neoplastic cells and it is specifically expressed in proliferating cell nuclei. This specific antibody recognizes PCNA protein, which is at its maximum level in late G1 and S phases of proliferating cells.^{17,18} However, there has been controversy about the diagnostic impact of PCNA. Ray et al¹⁹ demonstrated that the PCNA index was correlated with necrosis, high grade atypia, cellularity and mitotic rate. In contrast, Sbasching et al²⁰ did not find correlation of PCNA expression with survival in GIST. In order to find a more reliable index for the early diagnosis of PCa, our group investigated the changes of ratio value of Ki-67 and PCNA expression in different tissues. We found that the ratio of two markers increased following the grading of PCa and there were differences in PCa, BPH and normal prostate tissues.

In conclusion, this study suggests that Ki-67 and PCNA as proliferating markers should be combined and their ratio may be more effective and more accurate in the early diagnosis of PCa.

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