GENETIC ALTERATIONS OF ANDROGEN RECEPTOR GENE AND P53 BY FLUORESCENCE, IN SITU HYBRIDIZATION AND IMMUNODETECTION OF BCL-2 IN PROSTATIC INTRAEPITHELIAL NEOPLASIA AND CARCINOMA

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ABSTRACT

Objective: p53 and androgen receptor gene changes and bcl-2 expression in prostatic intraepithelial neoplasia (PIN) and adenocarcinoma were studied. The genetic associations of PIN and adenocarcinoma as detected by FISH and immunohistochemistry were discussed.

Material and Method: The study group consisted of 18 prostate cancer cases treated by radical prostatectomy. The p53 and androgen receptor gene changes at chromosomal level were studied using the FISH technique. Also, immunohistochemistry was used to search for bcl-2 expression.

Results: Of the 18 cases studied by FISH, it was found that in benign areas AR gene amplification was 78% (14/18) and 100% disomy with wild type p53, in PIN areas AR gene amplification was 33% (6/18), p53 amplification 28% (5/18), in carcinomas AR gene amplification was 39% (7/18), and p53 amplification 28% (5/18). We observed the bcl-2 immunexpression mainly in basal ductal cells in benign, PIN and carcinoma areas.

Conclusion: The results of our study suggest that AR gene amplification is significantly higher in benign areas compared to carcinomatous foci and that the presence of AR gene amplification is associated with the tumour stage. PIN is the most likely precursor of prostatic carcinoma.

Key Words: Androgen receptor gene, p53, FISH, Prostate carcinoma

INTRODUCTION

Prostate cancer is the most common cancer amongst men and second only to lung cancer as a cause of death from cancer (1). The Gleason histologic grade is an important prognosticator closely tied to tumour volume, capsular penetration, seminal vesicle involvement, lymph node metastases, clinical and pathologic stage and patient survival (2). Prostatic intraepithelial neoplasia (PIN) is a microscopic finding, and high grade PIN is considered to be the most likely precursor of prostate cancer (3,4). Today the assessment of the malignant potential of prostatic cancer is mainly based on morphological grading, staging and evaluation of

the tumour volume. Although significant differences in tumour behaviour can be shown between groups of patients concerning these various factors, their reliability as predictors of the course of any individual tumour is limited. Identification of markers that assist in forecasting the behaviour of a specific patient's tumour would be very useful.

Androgen receptor (AR) plays a central role in mediating the biological effects of androgens. Much research activity has been focused on searching for molecular defects of the AR gene in hormone refractory prostate cancer (1).

Bcl-2 functions as a cell death suppressor. It provided the first clue that altered gene expression could enhance cell survival without affecting cell proliferation (5). The expression of Bcl-2 in hormonal responsive organs is complex. In adult prostate, Bcl-2 expression appears to be inversely related to androgen stimulation, being mainly restricted to basal ductal cells that are resistant to androgen stimulation and androgen regulated apoptosis (5). Secretory cells sensitive to androgen treatment are largely Bcl-2 negative (6, 7). Mutations in the P53 gene represent the most common genetic changes in human malignancies. The P53 gene is located on chromosomal locus 17p13 and encodes for a 53kD nuclear phosphoprotein that functions as a cell-cycle regulation protein and inhibits the progression of genetically damaged cells through the S-phase (8).

Quantitative DNA analysis by flow or image cytometry has been reported to offer additional prognostic information for prostatic carcinoma but fluorescence in situ hybridization (FISH) is found to be more sensitive for the determination of gene and chromosome dosage in solid tumours (9, 10). Furthermore, FISH allows precise histopathologic correlation of normal epithelium, premalignant lesions and carcinoma within a single section, including the evaluation of intratumoral heterogenity.

We studied p53 and androgen receptor gene changes at chromosomal level with the FISH technique. We also searched for bcl-2 expression by immunohistochemistry. We discussed the genetic associations of PIN and adenocarcinoma detected by FISH and immunohistochemistry.

MATERIAL AND METHOD

The study group consisted of 18 prostate cancer cases treated by radical prostatectomy. Representative formalin-fixed, paraffin-embedded tumour blocks were selected by histopathological examination of haemotoxylin and eosin stained slides. We selected a representative block for each case which had high grade PIN and benign areas besides the carcinomatous foci on the same section and each foci were mapped directly on each slide. The clinical data were collected from patient records. Tumours were staged according to TNM (1999) classification and histological grading was done according to World Health Organizations (WHO) recommendations. The clinical data are presented in Table I.

DNA Probes

The following locus specific identifier (LSI) DNA fluorophore-labeled probes were used for the prostatectomy specimens: LSI Androgen Receptor Spectrum Orange (Vysis, Lot/Ch. B:22389), LSI p53 Spectrum Orange (Vysis, Lot/Ch.B:25076). LSI/WCP hybridization (Dextran sulphate, formamide, SSC).

Fluorescence in situ hybridization

We performed fluorescence in situ hybridization analysis on 5 μm. thin serial sections of the selected cases. The sections were de-waxed in xylene (2x5 min), rinsed in 100% ethanol (2x5 min) and air dried. Prior to FISH, the slides were placed in a coplin plastic jar filled with PBS for 5 min and dehydrated in ethanols (70%, 80%, 90%). After air drying, the sections were pre-treated by 2X standard saline citrate (SSC) at 37°C for 30 minutes, followed by 0.01 NHCL + 100μl pepsin treatment at 37°C for 10 min. Afterwards, 2X SSC treatment was applied at room temperature for 5 min. Subsequently, sections were placed in a coplin plastic jar filled with denaturation solution (70% formamide/2XSSC) at 73°C for 5 min, followed by dehydration in ethanol (70%, 80%, 90%). Probe mixture was prepared as follows: 7μl LSI hybridization buffer + 1μl probe + 2μl purified H$_2$O. After centrifugation, the probe mixture tube was centrifuged, then placed in a 73°C water bath for 5 min. and warmed to 45°C. Probe mixture was applied to the target area on slides, they were then placed in a pre-warmed and

Table I.: Clinical and histopathologic features of the cases in carcinomatous, intraepithelial neoplastic and benign areas

Bx	Rec.	Stage	SM	VS	GS	WHO	AR Ca PIN B	P53 Ca PIN B	Bcl-2 Ca PIN B
1	+	2 (3c)	+	+	9	3	D D A	D D D	- - -
2	+	2 (3c)	+	+	7	2	D D A	D D D	- - -
3	+	2 (3c)	+	-	8	3	D D A	D D D	+ + +
4	+	2 (3c)	+	+	9	3	A A A	D D D	- - -
5	+	2 (3c)	+	+	8	2	D D D	A A D	- - -
6	+	1 (2b)	-	-	7	2	D D A	D D D	+ + -
7	+	2 (3c)	-	-	9	3	A D D	D D D	+ - +
8	+	2 (3c)	+	+	7	2	A D D	A A D	- - -
9	-	1 (2b)	-	-	4	2	D D A	D D D	- + +
10	-	1 (2a)	-	-	3	1	D D A	D D D	+ + +
11	-	2 (3a)	+	-	7	2	D A A	D A D	+ + -
12	-	2 (3a)	+	-	6	2	A A A	A A D	- - -
13	-	2 (3a)	-	-	7	2	A D D	A D D	- - -
14	-	2 (3b)	+	-	7	2	A A A	D D D	- - +
15	-	2 (3c)	-	+	5	2	D A A	A D D	- + -
16	-	1 (2c)	-	-	4	1	D D A	D D D	+ + +
17	-	1 (2c)	-	-	5	1	D D A	D D D	- - -
18	-	2 (3a)	+	-	5	2	A A A	D A D	+ + +

SM: Invasion in surgical margin, VS: Invasion in Vesicula Seminalis GS: Gleason Score	D: Diploid, A: Aneuploid
AR: Androgen Receptor Gene, P53: p53 wild type,	Ca: Carcinoma PIN: Prostatic intraepithelial neoplasia B: Benign
bcl-2: bcl-2 protein expression by immunohistochemistry	Rec: Recurrence

humidified chamber in a 37°C incubator for 12 hours. After hybridization, the slides were washed in 0.4XSSC/0.3%NP-40 mixture for 2 min and then in 0.4XSSC/0.1%NP-40 at 73°C for 2 seconds. The slides were counterstained with 4,6 diamindino-2-phenylindole (DAPI) and the coverslip was applied.

Evaluation

An Olympus BX 40 fluorescence microscope was used for scoring signal copy numbers from a minimum of 100 nuclei per hybridization for each probe from the previously mapped carcinomatous, PIN and benign foci. The number of epithelial nuclei containing zero, one, two, three, four, five and more signals was recorded for each probe. The criteria for FISH anomalies were defined as previously explained (11).

Immunohistochemistry

Immunohistochemistry was performed by applying the Biotin Strepteavidin Amplified system on the serial sections of the cases. The bcl-2 mouse monoclonal antibody (BioGenex AM 287-5M) was applied for 30 min. at room temperature. Diaminobenzidyn (DAB) was used as a chromogen. The sections were counterstained with Mayer's haemotoxylin and small cell lymphoma was used as a positive control. Benign, PIN, carcinomatous foci in all immunostained slides were evaluated as the presence (+) or absence (-) of bcl-2 immunexpression.

Statistical Analysis

Statistical analysis was performed using GraphPad InStat version 2.04a (GraphPad Software U.S.A.). Clinicopathological correlations were analysed by Paerson's Chi-Square, Student's T-Test and One Way Anova test. The significance was $p < 0.05$.

RESULTS

Patients ranged in age from 53 to 70 years (mean 62 years). Pathological stages included T1N0M0 (5 cases), T2N0M0 (13 cases). Table I summarises the tumour, the pathologic stage and grade of the tumour, the recurrence status of

the patient, AR gene and wild type p53 ploidy results with fluorescence in situ hybridization and bcl-2 protein immunexpression data for the individual cases. Of the 18 cases studied by FISH, we found AR gene amplification in benign areas (Fig. 1) was 77.7% (14/18) and 100% disomy with wild p53, in PIN areas it was 33.3% (6/18), p53 amplification 27.7% (5/18), in carcinomas it was 38.8% (7/18), and p53 amplification 27.7% (5/18) respectively. Statistically significant correlation was found only between AR gene amplification and stage (p<0.05). We could not reveal any statistical correation between wild type p53 and prognostic parameters such as stage, surgical margin invasion, vesiculo seminalis invasion, recurrence, WHO grade, Gleason score, AR and also bcl-2 immunexpression (Fig. 2). Also, bcl-2 immunexpression did not show any statistical correlation with the same prognostic parameters.
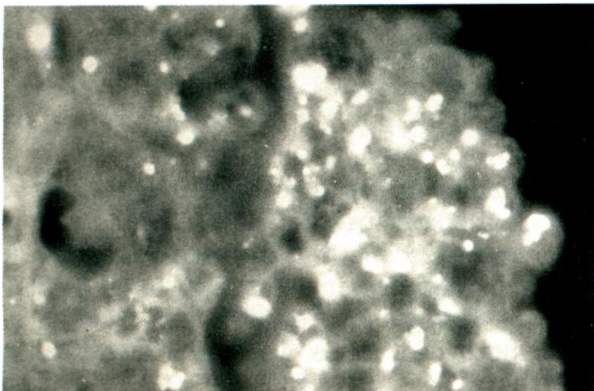

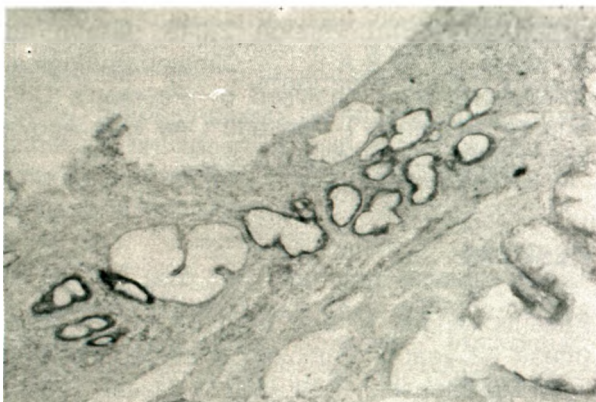**Fig.1 :** Androgen receptor gene amplification (X40)

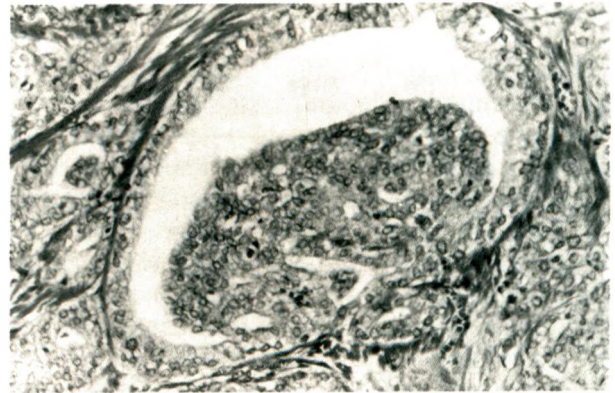**Fig.2 :** bcl-2 immunoexpression (X20)

Fig.3 : Intraepithelial neoplasia and carcinoma of the prostate (H&E X40).

DISCUSSION

Prostate cancer is second only to lung cancer as a cause of death from cancer (1). Androgen withdrawal constitutes the only effective form of systemic therapy for metastatic prostate cancer. The mechanisms of the appearance of androgen independent clones of tumour cells limit the effective control of the patient's disease (12). In our study we found that the presence of AR gene amplification is associated with the advanced stage of the tumour (p<0.05). Takedo et al, have demonstrated that carcinomas with a low Gleason score have a significantly higher AR than those with a high Gleason score and AR content has not been found to be correlated with the extent or the disease in stage D2 prostate carcinomas (14). AR concentration has been found to be lower compared to benign tissue and has no correlation with Gleason score in stage D2 prostate carcinomas but there has been correlation of p53 expression to Gleason score (15). AR gene amplification and PSA protein have been found to be correlated by FISH and immunohistochemistry (16). Our results are consistent with the previous studies, as AR gene amplification was shown to be significantly higher in benign areas (78%) compared to carcinomatous foci (39%). On the other hand, our results show discordance with studies which demonstrate that AR amplification is observed only in recurrent tumours during androgen deprivation therapy and in none of the untreated primary tumours (17-20).

In the literature, AR gene amplification is also found to be correlated with p53 immunostaining in hormone refractory recurrent prostate cancer (17). Our study group was composed of primary cancer cases, without any prior endocrine treatment and we could not reveal any statistically significant correlation between wild type p53 and AR gene amplification. A previous study reported intratumoural p53 heterogenity in a prostate carcinoma sample with some areas of the tumour having mutated p53 and others having wild type p53 (21). P53 mutations have been shown in 19% of benign prostatic hyperplasia (BPH) and 2 patients with mutations in BPH tissue developed prostate cancer in 2-3 years. Thus it has been concluded that p53 mutation in BPH tissue may be a risk factor (22).

In contrast to this study we found 100% disomy with p53 in benign areas of the prostate carcinomas. Positive association between p53 immunreactivity and higher Gleason grade has been demonstrated (23). We could not demonstrate any statistical correlation between wild type p53 and other prognostic parameters such as tumour stage and grade, positive surgical margin, vesiculo seminalis invasion, recurrence, WHO (World Health Organization) grade, Gleason grade, AR and also bcl-2 immunexpression.

Several hypotheses on the molecular mechanisms of tumour recurrence have been suggested. These include overexpression of the bcl-2 oncogene (24-26), activating mutations, amplification and overexpression of the AR gene (17, 18). bcl-2 amplification has been shown by increased copy number by FISH; however no high level amplifications have been found in hormone refractory recurrent prostate carcinomas (27). We could not reveal any statistical correlation between bcl-2 immunexpression and tumour stage, positive surgical margin, vesiculo seminalis invasion, recurrence, WHO grade, Gleason grade, AR and also wild p53 amplification. We mainly observed the bcl-2 immunexpression in basal ductal cells, which is consistent with the literature (5-7, 28, 29).

We demonstrated that PIN and prostatic carcinoma foci have a similar proportion of genetic changes for AR gene, but foci of carcinoma have more alterations. This supports the hypothesis that PIN is the most likely precursor of prostatic carcinoma (3, 4, 11, 13). The results of our study suggest that the presence of AR amplification is associated with advanced disease and PIN is the most likely precursor of prostatic carcinoma.

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