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Research Article

Identification of proteins in human prostate tumor material by two-dimensional gel electrophoresis and mass spectrometry

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Abstract. Protein patterns in cells collected from benign prostatic tissues and prostate carcinomas were analyzed using two-dimensional polyacrylamide gel electrophoresis and mass spectrometry. Polypeptide expression was evaluated by computer-assisted image analysis (PDQUEST). Proteins expressed by prostate tumors were identified via in-gel digestion and subsequent matrix-assisted laser desorption/ionization mass spectrometry. In addition to cytoskeletal and mitochondrial proteins, a 40-kDa protein was identified as prostatic acid phosphatase (PAP). PAP expression decreased approximately twofold between benign and malignant tissue. Increased expression of heat shock protein 70 and decreased expression of tropomyosin 1 were also observed in the malignant tissue. The analysis of prostate material by two-dimensional gel electrophoresis and mass spectrometry shows that particular proteins are of interest as markers of disease.

Key words. Prostatic carcinoma; benign prostatic tissue; two-dimensional gel electrophoresis; mass spectrometry.

Prostate cancer is an epithelially derived form of malignancy showing an increased frequency in the Western world [1]. Because of the relative lack of clinical symptoms, prostatic neoplasia are often detected at a late stage in tumor development. Important tools in prostate cancer diagnosis are serum markers such as prostate-specific antigen (PSA) and prostatic acid phosphatase (PAP) [2, 3]. However, diagnosis based on PSA levels in the range of 4–20 ng PSA/ml serum does not differentiate neoplasia from hyperplasia [2, 4], and when high PSA levels are detected, the tumor often displays extracapsular progression, which means poor prognosis [5]. Therefore, efforts should be made to find sensitive and specific diagnostic markers which can discriminate between tumor subtypes.

In this study we employed two-dimensional polyacrylamide gel electrophoresis (2D/PAGE) to analyze protein expression patterns in specimens from prostatic carcinoma and benign prostatic tissue. The aim was to find a correlation between altered tissue morphology and polypeptide expression which could complement the diagnostic markers already in use, and to begin a

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wider scan of the prostate proteome for carcinoma-specific markers.

Materials and methods

Histopathology. Sixteen prostate glands from patients aged 56–80 years (average 66.5 years) were obtained via radical prostatectomy, and analyzed by 2D/PAGE following both macro- and micropathologic anatomic diagnosis. The prostatectomy specimens were immediately transported on ice to the pathology department. The glands were divided into two halves by a horizontal section and slices of approximately $10 \times 10 \times 3$ mm were cut. Samples were taken from tumor-suspicious areas, usually in the dorsolateral part of the peripheral zone. Benign tissue was collected from hyperplastic nodules in the transition zone or from the contralateral peripheral zone. The samples were sectioned in two parallel slices. One slices was embedded in Histocare gel (Amedic, Sweden) and snap frozen; the other was used for 2D/PAGE. A 4- to 5-μm-thick section was taken from the snap-frozen sample and stained with hematoxylin-eosin. The tumor was confirmed and graded according to the Gleason system (table 1). The prostatectomy specimens were formalin fixed, sliced 4 mm thick, and embedded in standard cassettes. The blocks were processed routinely and the sections stained with hematoxylin-eosin. The tumor was outlined on the slides and the Gleason score was assessed. Apparently homogeneous samples, ten carcinomas and nine benign tissues, were selected for further analysis. In three cases, both carcinoma and benign tissues were collected from the same gland. In general, micropathological tumor borders are diffuse and a complete correlation between macro- and micropathology is, therefore, difficult.

2D/PAGE and protein pattern analysis. Tumor material was prepared according to a non-enzymatic method [6], with slight modifications [7]. Briefly, cells were scraped from macroscopically evaluated non-necrotic areas using a scalpel and collected in medium supplemented with antiproteases. Viable cells were selected for 2D/PAGE work via Percoll gradient centrifugation and were lysed in a buffer containing 8 M urea, detergents, reducing agents, and protease inhibitors as described [6, 7]. The protein concentration was determined [8] and isoelectric focusing using immobilized pH gradient (IPG) strips was carried out by loading 75 μg protein in 340 μl (complemented with 2% v/v IPG-buffer 4–7 linear) onto a 17-cm IPG-strip 4–7 linear (BioRad). Focusing was performed to a total of 48.8 kVh using an IPGphor unit (20 °C). The second dimension was carried out in a 10–13% gradient SDS gel, and protein visualization was by silver staining [9]. Stained gels were scanned at 100-μm resolution using a Molecular Dynamics laser densitometer, and data were analyzed using the PDQUEST software (version 6.1, BioRad). Gel images were compared for qualitative and quantitative differences. Polypeptide quantities were calculated in ppm of the total integrated optical density.

Mass spectrometry. Proteins displaying variability in their expression pattern were selected for identification via mass spectrometry. One preparative gel loaded with

Table 1. Histopathologic characterization of tumor specimens used for 2D/PAGE analysis.

Sample	Diagnosis	Ploidy*	Gleason score†	Age (years)
1	adenocarcinoma	D	3+3	56
2	adenocarcinoma	D	4+3	68
3	adenocarcinoma	D/T	3+3	57
4	adenocarcinoma	D	3+3	66
5	adenocarcinoma	D	3+4	60
6	adenocarcinoma	D/T	4+3	72
7	adenocarcinoma	D	4+3	65
8	adenocarcinoma	D	3+3	64
9	adenocarcinoma	D	3+4	74
10	adenocarcinoma	D	3+3	56
11	benign tissue	D		79
12	benign tissue	D		67
13	benign tissue	D		60
14	benign tissue	D		66
15	benign tissue	D		77
16	benign tissue	D		60
17	benign tissue	D		72
18	benign tissue	D		80
19	benign tissue	D		62

* D diploid; T tetraploid.
† 1–5 indicates increasing amounts of epithelial cells without luminal differentiation, with 1 signifying no prostatic carcinoma and 5 signifying advanced prostatic carcinoma, as determined from two adjacent areas.

200 μg protein from a prostatic carcinoma sample was stained with Coomassie colloidal. In-gel digestion was carried out with trypsin [10] and digests were desalted using ZipTip (Millipore). Peptides were eluted in 70% acetonitrile/5% formic acid. The eluate was mixed 1:1 (v/v) with a saturated matrix solution of α-cyano-4-hydroxycinnamic acid in 30% acetonitrile/0.1% trifluoroacetic acid. Mass mapping of tryptic peptides was performed with a Voyager DE PRO MALDI-TOF (Applied Biosystems) or with a TofSpec-2E MALDI-TOF (Micromass) mass spectrometer, both equipped with a nitrogen laser (337 nm) and operated in the positive ion mode. Trypsin fragments of masses 842.50 Da and 2211.10 Da were used as internal standards for spectra calibration. Data generated were screened in databases using a mass tolerance ≤ 20 ppm. Programs used for mass mapping were MS-Fit (http://prospector.ucsf.edu) and ProFound (http://prowl.rockefeller.edu), or the licensed ProteinLynx Software (Micromass).

Results and discussion

Affected prostatic tissue from ten patients with prostatic carcinoma and from nine glands with benign prostatic tissues (table 1) was separated by 2D/PAGE (fig. 1A, B). The protein expression patterns were analyzed for qualitative and quantitative differences using the PDQUEST software. Proteins were identified via MALDI mass mapping and database searches. The patterns obtained were complex and showed interindividual variability even between samples with an identical macropathological diagnosis, although only specimens with a Gleason score of $3+3/3+4$ were used. This likely reflects the diffuse tumor borders in the tissues.

On average, 800 protein spots per gel were recorded. Following background subtraction and spot matching, polypeptides were quantified. Twenty-three proteins showed some differential values between malignant and benign tumors, and were selected for mass spectrometric identification (fig. 1C, table 2). They included proteins belonging to the heat shock family, enzymes, and structural proteins. In addition, a 40-kDa protein was identified as PAP.

Three proteins revealed a more clearly altered abundance in malignant compared to benign material. These proteins were identified as tropomyosin 1 (TM1), heat shock protein 70 (HSP70), and PAP. Decreased abundance of TM1 and PAP was detected concomitantly with increased expression of HSP70 in prostatic carcinoma.

TM1 is expressed by epithelial cells and fibroblasts, and is homologous to tropomyosins expressed in muscle cells [11]. Its function is not completely clear, although

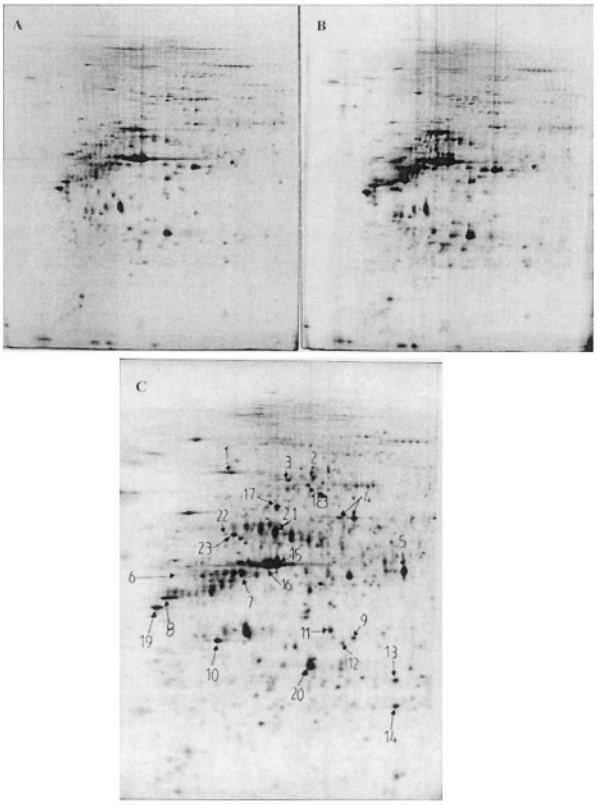

Figure 1. Silver-stained two-dimensional gel patterns of prostatic carcinoma (*A*) and benign prostatic tissue (*B*) from the same patient. Protein (75 μg) was loaded onto IPG-strips (4–7 linear pH gradient, 17 cm) followed by SDS/PAGE (10–13%). After staining, the spots numbered (*C*) were identified as listed in table 2.

it has been suggested that down-regulation of TM expression decreases cell stability (affecting both shape and motility) via an increased sensitivity of the microfilament to depolymerizing factors [12]. TM1 mRNA levels have consistently been found to be lower in transformed fibroblasts compared to normal cells [13], and are also low in malignant human breast lesions without lymph metastasis [14]. Also of importance is the fact that tissue morphology in prostatic cancer is altered, epithelial cells increasing at the expense of fibrotic stromal tissues. Therefore, it is difficult at the present stage to assess if the alteration detected in TM quantity depends on cellular down-regulation or altered gland composition.

HSPs stabilize protein structure, assist in protein translocation, and generally protect proteins from environmental stress which might otherwise result in denaturation or defective folding [15]. Increased levels of HSPs have been associated with malignancy [16] and, moreover, HSPs can apparently be used to predict recurrence of malignancy [17].

Table 2. Proteins identified from Coomassie stained 2D/PAGE (4–7 linear pH gradient, 10–13% SDS/PAGE; fig. 1C), by MALDI-MS. Accession number given is for SwissProt.

Spot number	Sequence coverage (%)	Peptides extracted	Accession number	Protein
1	39	22	P11021	78-kDa glucose-regulated protein
2	33	17	P38646	mitochondrial stress-70 protein
3	68	10	P08107	heat shock protein 70
4	40	18	P30101	probable protein disulfide isomerase ER-60
5	27	11	P15309	prostatic acid phosphatase
6	41	17	P08727	keratin type 1, cytoskeletal 19
7	16	5	P22680	cytochrome P450 7A1
8	18	8	P09493	tropomyosin α-chain
9	32	11	P09525	annexin IV
10	61	17	P08758	annexin V
11	49	14	P12429	annexin III
12	20	5	P31937	hydroxyisobutyrate dehydrogenase
13	46	10	P30084	enoyl-CoA hydratase, mitochondrial
14	21	5	P30048	mitochondrial thioredoxin-dependent peroxide reductase
15	30	13	P12718	actin, γ-enteric smooth muscle
16	35	13	P02571	actin, cytoplasmic 2
17	24	14	P10809	60-kDa heat shock protein, mitochondrial
18	27	15	P08107	heat shock protein 70 (fragment)
19	25	10	P06468	tropomyosin, fibroblast and epithelial muscle type (TM1)
20	28	10	P02570	actin, cytoplasmic 1 (fragment)
21	39	13	P17661	desmin
22	24	9	P06576	ATP synthase β-chain, mitochondrial
23	35	13	Q15084	probable protein disulfide isomerase P5

Of the remaining proteins identified, PAP may be noted. This protease is of interest because it is used as a marker for prostate cancer [3, 18]. In this study we found that the expression level of PAP is decreased approximately twofold in prostatic carcinomas.

Taken together, our findings are in agreement with results from other studies and indicate that the diagnosis of heterogeneous prostate tumor samples is improved when multiple markers are assessed.

Several factors limit direct analysis of malignant tumors. One is the diffuse micropathologic border of prostate tumors. In addition, sample amounts available for analysis are limited. This applies particularly to proteins directly responsible for neoplastic processes such as transcription factors, which are intrinsically present at low copy number. Despite these limitations, the present results show that particular proteins show distinct differences between malignant and benign tissues, and are of interest as marker proteins for disease.

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