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Advances in Brief

Tumor Progression Is Accompanied by Significant Changes in the Levels of Expression of Polyamine Metabolism Regulatory Genes and Clusterin (Sulfated Glycoprotein 2) in Human Prostate Cancer Specimens[1]

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Abstract

Using Northern blotting, the expression levels of the genes for polyamine metabolism regulatory proteins and clusterin have been measured in a series of 23 human prostate cancers (CaPs) dissected from radical prostatectomy specimens. Patient matched, nontumor tissue was dissected from benign areas of the gland. The results indicate that transcripts encoding ornithine decarboxylase (ODC), ODC antizyme, adenosylmethionine decarboxylase, and spermidine/spermine N^1-acetyltransferase (SSAT) were significantly higher, whereas clusterin (sulfated glycoprotein 2) mRNA was significantly lower in tumors compared with the benign tissue. All mRNA levels were compared with those of histone H3 and *growth arrest-specific gene 1*, markers of cell proliferation and cell quiescence, respectively, and *glyceraldehyde 3-phosphate dehydrogenase*, a housekeeping gene. In poorly differentiated and locally invasive CaPs and in tumors with unfavorable prognosis or total prostate-specific antigen (PSA) levels >10.0 ng/ml at diagnosis, an overall increase in the levels of H3 mRNA and a decrease in *growth arrest-specific gene 1* mRNA was detected, indicative of higher proliferation activity, whereas the differences in expression levels for the polyamine metabolism and clusterin genes were higher. ODC and SSAT changes were positively correlated in normal tissue but not in high-grade cancer, whereas ODC antizyme and SSAT changes were positively correlated in more malignant CaPs but not in normal tissue. Tumor classification based on the changes in expression levels of all of the genes studied could be correlated to differentiation grade and local invasiveness classification systems in 72.2 and 83.3% of the cases, respectively. In a 1-year follow-up period, three patients whose CaPs ranked as less aggressive according to clinical staging, but classified as advanced cancers with the proposed molecular classification, showed increases in total PSA levels, indicative of tumor relapse. Thus, molecular classification, based on gene expression, may enhance the available prognostic tools for prostate tumors.

Introduction

The aliphatic polyamines putrescine, SPD,[3] and SPM are necessary for normal and pathological cell growth, and increases in the levels of these polycations have been associated with cell proliferation and cell transformation induced by growth factors, carcinogens, viruses, or oncogenes (1, 2). In the prostate gland, polyamines are present at high levels and are positively controlled by androgens. Innumerable studies have tried to relate changes in polyamine levels, in tissue or body fluids, to tumor growth but, although there is no doubt that polyamines are necessary, the changes measured were not specific enough to be used as good molecular markers of malignant progression (1).

ODC (EC 4.1.1.17), the first and rate-limiting enzyme of polyamine biosynthesis, was shown to be critical in cell transformation and suggested to be a proto-oncogene (3). ODC activity is regulated by a unique protein, OAZ, that, after being induced by high levels of intracellular polyamines, binds and inhibits ODC, accelerating its degradation by the 26S proteasome system; OAZ also inhibits polyamine uptake from the extracellular compartment and may accelerate polyamine release from the cells (4). The second enzyme of polyamine biosynthesis, also a rate-limiting one, is AdoMetDC (EC 4.1.1.50), required for the production of SPD and SPM from putrescine and adenosylmethionine. A catabolic pathway (retroconversion pathway), which is regulated by SSAT (EC 2.3.1.57), leads to polyamine degradation or excretion (5). SSAT is induced by polyamine overaccumulation, thus taking part, together with OAZ, in the mechanisms that control intracellular homeostasis of these amines. Very recently, we have shown that expression of the genes coding for ODC, OAZ, AdoMetDC, and SSAT is cell cycle related in human dermal fibroblasts, leading to coordinate variations in the intracellular concentrations of polyamines, with cyclical phases of depletion and accumulation during cell cycle progression (6).

Clusterin (also named SGP-2 and many other acronyms) is a heterodimeric glycoprotein present in most animal tissues and body fluids (7). It is overexpressed in the regressing rat prostate 2–10 days after androgen ablation (8). In general, expression of clusterin is down-regulated during cell proliferation (9) but up-regulated under conditions inducing cell suffering, cell atrophy, or organ involution (7, 8), in recent data from our laboratory, is induced in quiescent cells (10). Clusterin expression thus shows an inverse pattern to that of ODC in prostate. A role in the process of inhibition of apoptosis and induction of cell survival has been proposed for this gene (11), which may bear some relationship with the selection of the transformed phenotype and acquisition of hormone independence.

Given the complexity of the regulation of polyamine metabolism, we set out to overcome the limitations of previous studies by measuring the expression levels of the entire set of genes that regulate polyamine metabolism. The levels of their mRNAs were measured in benign and malignant areas, dissected from the same radical prostatectomy specimens, together with clusterin, the expression patterns of which have been studied extensively in our laboratory (8–10), histone H3, a marker of cell proliferation, the mRNA of which accumulates in S phase of the cell cycle (12), and Gas1, involved in growth suppression and maintenance of the quiescent state (13). Finally, for com-

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[3] The abbreviations used are: SPD, spermidine; SPM, spermine; ODC, ornithine decarboxylase; OAZ, ornithine decarboxylase antizyme; SSAT, spermidine/spermine N^1-acetyltransferase; AdoMetDC, adenosylmethionine decarboxylase; SGP-2, sulfated glycoprotein 2 (clusterin); *Gas1,* growth arrest-specific gene 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PSA, prostate-specific antigen; CaP, prostate cancer.

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parison, the levels of the mRNA coding for GAPDH, a typical house-keeping gene, was also detected in the same specimens.

The data allowed a comparison, not only of the levels of the single transcripts in normal and tumorous tissues of different grade of prostatic malignancy but also between the patterns of expression of the whole set of genes, revealing the characteristic expression profiles that may establish during neoplastic transformation. Furthermore, by appropriate statistical analysis, we have shown significant correlations between expression levels of the genes studied, which may help to understand their involvement in CaP progression, and provide molecular markers of malignancy that may complement those in current clinical use.

Materials and Methods

Patient Treatments. The study was conducted in prostate surgical specimens from a group of patients all subjected to the same standard protocol of long-term chemical androgen ablation, *i.e.,* androgen suppression therapy for 3 months before surgery, according to a standard protocol: 200 mg/day of ciproterone acetate, plus a single dose (3.75 mg) of the luteinizing hormone releasing hormone agonist leuproreline for the first month of treatment, followed by leuproreline alone for the next 2 months. This investigation was performed after approval by the local Human Investigation Committee and after obtaining informed assent from the patients involved in the study. Total PSA levels were determined at diagnosis.

Patient Samples. Radical prostatectomy samples were obtained from 23 patients with tumors graded from Gleason grades 1 to 5 (Gleason scores 2 to 9). Immediately after total prostatectomy, a specimen of about 0.5 cm^3 in size was excised out from the cancerous portion of each individual gland. A second well separated area of benign (normal) tissue was also dissected. Both specimens were quickly frozen on a flat glass surface at $-80°C$, covered with dry ice powder, to preserve the tissue orientation and to generate a suitable cutting surface for the cryostat. Starting from the top surface, alternate 14-μm sections and tissue slices of about 200 mg were made from both the cancerous and normal specimens. The 14-μm tissue sections were stained and used for the histopathological and morphological characterization of the thick tissue slice between the two 14-μm sections. Two thick slices were obtained from each specimen. Only normal specimens with no sign of benign prostatic hypertrophy or tumor invasion and a similar relative epithelial cell and stromal content to that of the cancerous counterparts were used as controls. One of the two 200 mg slices from each specimens (normal or cancerous) was used for total RNA extraction; the other one was used for Western blot analysis and enzyme assays.

Northern Hybridization Analysis. Total RNA was extracted from frozen human tissue specimens using RNAfast (Molecular Systems, San Diego, CA). Ten-μg aliquots were then electrophoresed on a 1% agarose-formaldehyde gel, blotted onto Hybond-N nylon membranes (Amersham Italia S.r.l.), and then hybridized to the specific cDNA probes. The specific cDNA probes were previously purified and labeled by random priming [^{32}P]dCTP incorporation as described previously (14). The Gas1 cDNA, kindly donated by Dr G. Del Sal (C. I. B. Consorzio Interuniversitario Biotecnologie, Trieste, Italy) (13), and the histone H3 probe, kindly donated by Dr G. Stein (Department of Biochemistry and Molecular Biology, University of Florida College of Medicine, Gainesville, FL) (15), were purified, labeled, and used following the same procedures. Quantitation of the autoradiograms was obtained by densitometric scanning using a LKB Ultrascan XL densitometer.

Statistical Analysis. The statistical significance of the differences between the mean values of the abundance of each mRNA, in the normal and cancerous portions of the prostate gland, was determined by paired comparison t test analysis (two-tailed). The same analysis was applied to assess the statistical significance of the above differences, between normal and cancerous portions, after grouping the data as a function of the differentiation grade, tumor localization, prognosis, and total PSA level at diagnosis. The Pearson r test analysis (two-tailed) was used to assess significant correlations (positive or negative) between the values expressing the mRNA levels of each gene, determined either in normal or cancerous tissues. The logistic regression analysis was used for the classification of the tumors as a function of the levels of expression of group of genes in the cancerous part of the prostate. This was compared with the classification based on the differentiation grade or tumor localization.

Results and Discussion

Tumor growth is a dynamic process, the progression of which is characterized, at any given time, by the relative number of normal and tumor cells undergoing cell proliferation, cell death, and cell quiescence. In the present report, we have set out to determine the levels of expression of genes that are involved in these processes.

Fig. 1*A* shows a representative Northern blot in which the expression levels of H3 and Gas1 mRNAs are inversely related in matched cancerous and normal specimens. For example, in the Gleason score 2 tumor, *Gas1* was up-regulated but *H3* was down-regulated, suggesting that, in less aggressive CaPs, overall cell proliferation is probably lower than in the normal tissue. The opposite was true in the Gleason scores 5 (Gleason grade: 3+2) and 8 (Gleason grade: 5+3) tumors, where *Gas1* was down-regulated, whereas *H3* was overexpressed with respect to normal tissue. These data are consistent with the positive correlation between CaP progression and proliferation activity that was shown previously with Ki-67/MIB1 or PCNA/cyclin as specific markers of cell proliferation (16–18).

The relative levels of the mRNAs coding for the regulatory proteins of polyamine metabolism ODC, AdoMetDC, SSAT, and OAZ, together with SGP-2 and GAPDH as determined in two of the above specimens (Gleason scores 2 and 8) are shown in Fig. 1*B*. In the less aggressive tumor, with lower proliferative activity (Gleason score 2), *ODC* is up-regulated, and this is accompanied and perhaps compensated by the increase in OAZ mRNA. The AdoMetDC transcript is not induced, and a minor increase of the mature SSAT mRNA form (1.3–1.5 kb) is evident, probably leading to a slight increase in polyamine degradation and excretion. This pattern, exhibited by a well-differentiated cancer, is consistent with the hypothesis that ODC induction, which should be balanced by activation of the regulatory steps of the polyamine metabolism to prevent detrimental over accumulation of these polycations, is an early event during cell transformation (1). In contrast, in the actively proliferating Gleason score 8 CaP (Fig. 1*B*), the induction of ODC does not seem to be counterbalanced by *OAZ* overexpression. Equally, the levels of the AdoMetDC transcript are dramatically higher in this specimen, and the increased accumulation of higher polyamines (SPD and SPM), which inevitably would follow the induction of the two biosynthetic enzymes, may be partly balanced by *SSAT* overexpression. This would result in intracellular polyamine concentrations capable of supporting a high rate of cell proliferation without jeopardizing cell survival.

Clusterin (SGP-2) is down-regulated both in Gleason scores 2 and 8 CaP specimens (Fig. 1*B*), showing that changes in the expression of this gene are both the inverse of *ODC* and are among the earliest events of prostate tumor development.

The densitometric values obtained from autoradiograms of Northern hybridization experiments performed in all normal and cancerous specimens ($n = 23$) were then analyzed by t $test_{[p.c.]}$, a paired comparison t test. Although not all of the mRNAs could be titrated in all of the specimens studied, the number of determinations varied between 18 (for H3) to 23 (for OAZ). The mean values for the abundance of each transcript, in normal and cancerous portions of the same gland, were calculated and are shown in Fig. 2. The mRNA levels of each of the regulatory proteins of polyamine metabolism were significantly higher in the tumor than in the normal portion of the gland (ODC: $t = -2.852$, $n = 20$, $P = 0.008$; OAZ: $t = 2.220$, $n = 23$, $P = 0.038$; AdoMetDC: $t = 2.462$, $n = 21$, $P = 0.023$; SSAT: $t = 2.767$, $n = 22$, $P = 0.009$), confirming a very recent report showing that ODC activity and protein are significantly higher in

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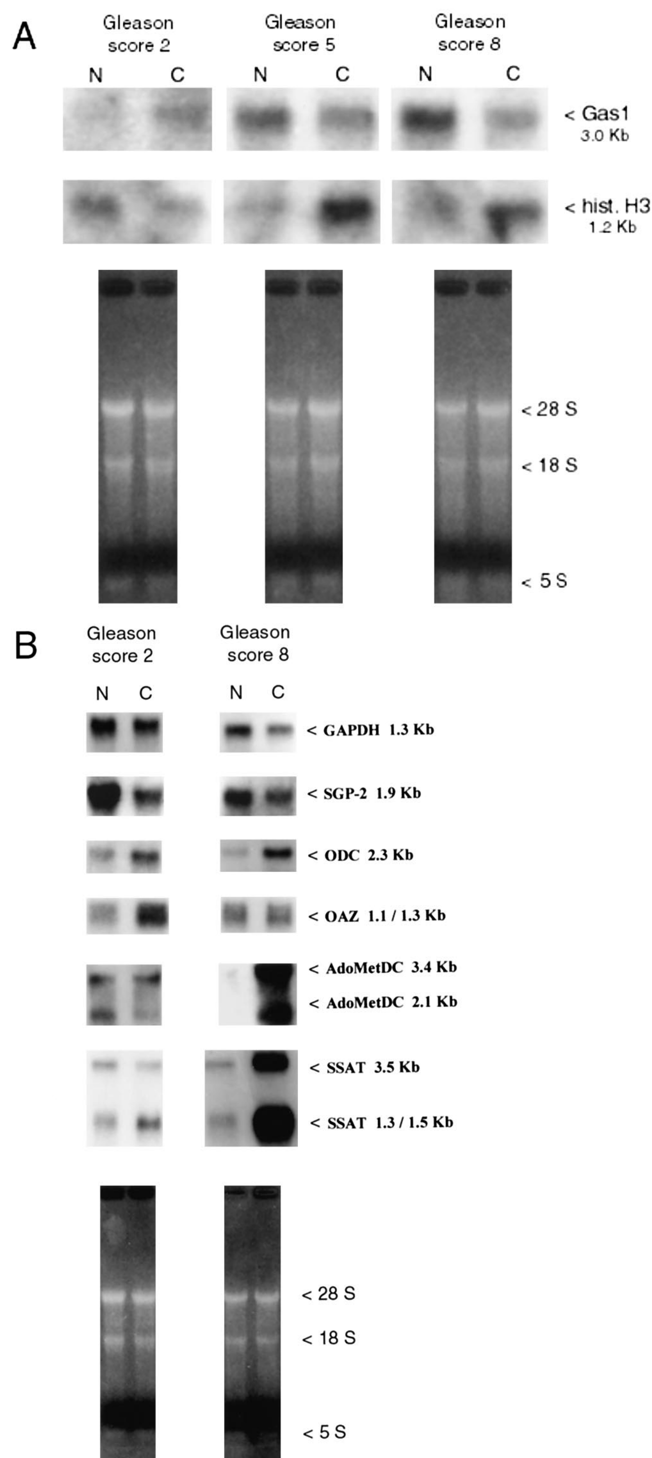

Fig. 1. Representative Northern blot autoradiograms of total RNA extracted from low (2 and 5) and high (8) Gleason score human CaP specimens (*C*), compared with the benign counterpart (N) from the same gland. The specific radioactive probes used for the hybridization and the size of the specific mRNAs detected are indicated. Ethidium bromide staining of the same RNA samples is shown for comparison (10 μg of total RNA were loaded on each lane and resolved by electrophoresis). *A,* histone *H3* and *Gas1* mRNAs; *B,* mRNAs coding for the regulatory proteins of polyamine metabolism, ODC, AdoMetDC, OAZ, SSAT, and clusterin (SGP-2). *GAPDH* mRNA is also shown for comparison.

human CaP as compared with paired benign tissue (19). Conversely, the level of clusterin transcript in CaP specimens was significantly lower than in normal tissue ($t = -2.719$; $n = 20$; $P = 0.009$).

To confirm that the changes in mRNA levels were reflected at the protein level, the enzymatic activities of the degradative SSAT and biosynthetic AdoMetDC (essential for SPD and SPM production), when assayed in a subset of the specimens, paralleled the different abundance of the corresponding transcripts in tumor and normal tissues (not shown). In addition, lower levels of clusterin protein were detected in cancerous than in normal tissue by Western blot analysis (not shown), again in agreement with the decrease of clusterin mRNA levels in tumor tissue.

A trend toward higher histone H3 mRNA levels, in 61% (11/18) of the patients, and lower Gas 1 mRNA levels, in 68% (15/22) of the patients, in CaP tissue compared with the matched normal tissue was also detected. The differences approached but did not reach statistical significance (H3: $t = -1.948$; $n = 18$; $P = 0.068$; Gas1: $t = 1.104$; $n = 22$; $P = 0.540$). Taken together, these data suggest that in CaPs, more cells are proliferating and fewer are quiescent, which is further supported by the decrease in SGP-2 expression that we have shown previously to occur after application of a proliferation stimulus (9, 10). Higher levels of GAPDH mRNA detected in cancer with respect to the normal portion of the gland were not significant ($t = 1.314$; $n = 22$; $P = 0.203$), as in only 55% of the patients (12 of 22) was GAPDH expression higher in cancerous than in normal tissue.

The mean values of the mRNA accumulation in CaPs were next analyzed as a function of the differentiation grade (Gleason grade 1–2, well differentiated; grade 3, moderately differentiated; grade 4–5, poorly differentiated). In poorly differentiated cancers, the levels of ODC, AdoMetDC, SSAT, Gas1, and SGP-2 transcripts, expressed as percentages of the mean values determined in the normal counterparts (Fig. 3*A*), exhibited the largest significant differences (t test$_{[p.c.]}$). In moderately differentiated tumors, only *ODC* was significantly overexpressed, whereas H3 mRNA reached its highest level, not far from statistical significance ($t = -2.378$; $n = 6$; $P = 0.063$). Paradoxically, in the well-differentiated cancers, the expression profile for *H3* and *Gas1* appeared to be opposite of that observed in poorly differentiated tumors: *i.e.,* H3 mRNA accumulation was lower and Gas1 mRNA

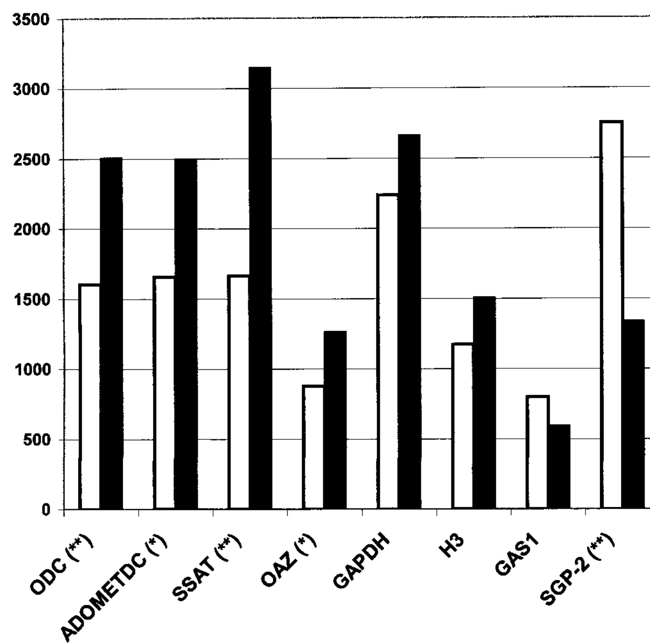

Fig. 2. Mean values of the relative abundance of the mRNAs studied in normal and cancerous human prostate specimens from the same gland ($n = 23$). □, normal; ■, cancerous. The specific mRNA radioactive signals, obtained by Northern blot analysis as in Fig. 1, were quantified by densitometric scanning of the autoradiograms. The data obtained for each transcript were then subjected to t test$_{[p.c.]}$ analysis: (*), $P < 0.05$; (**), $P < 0.01$.

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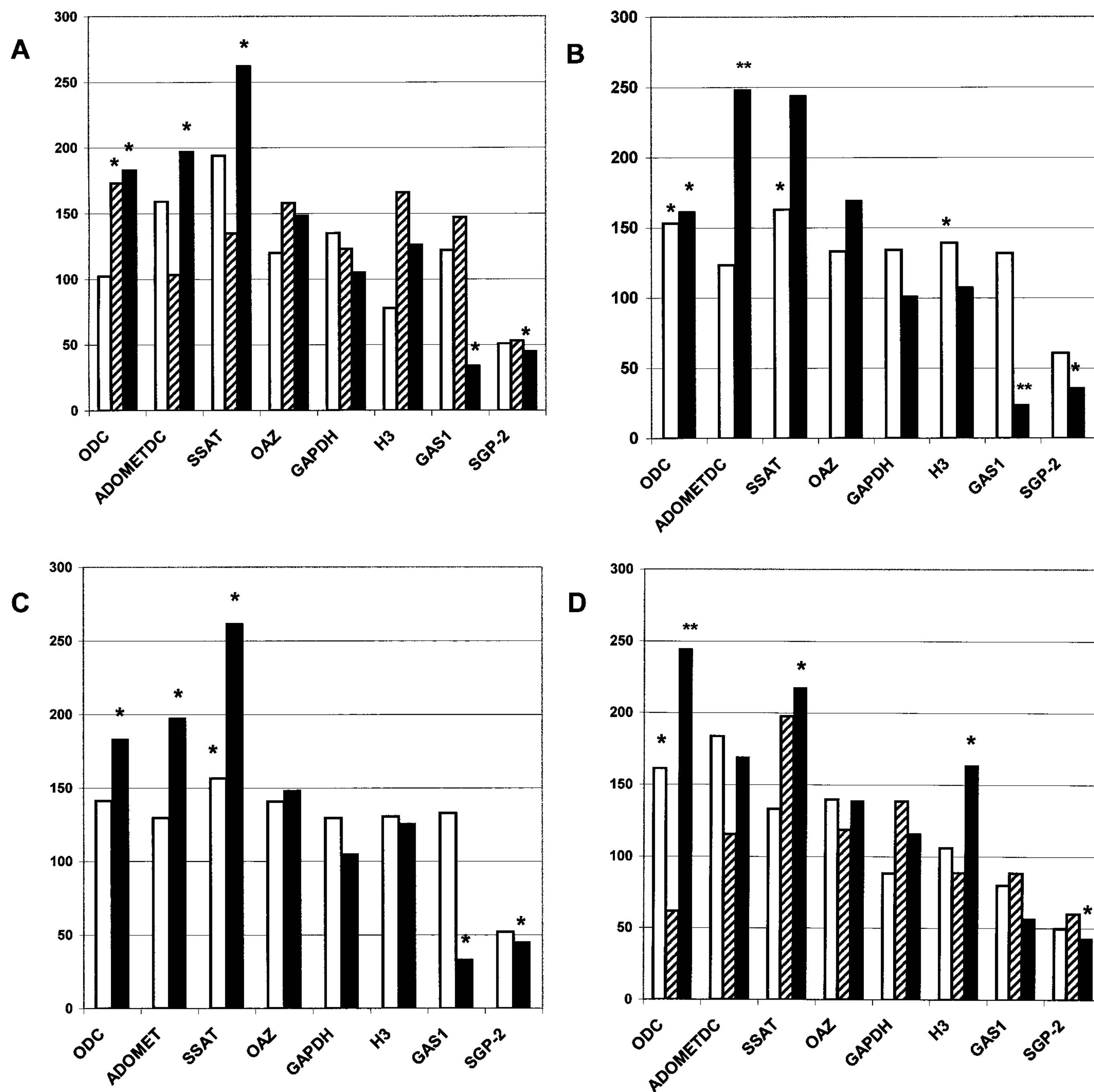

Fig. 3. The same data of Fig. 2 were analyzed after grouping the specimens as a function of: *A*, differentiation grade: □, well differentiated (Gleason grades 1–2; $n = 8$); ▨, moderately differentiated (Gleason grade 3; $n = 7$); ■, poorly differentiated (Gleason grades 4–5; $n = 8$); *B*, tumor localization: □, organ-confined CaP ($n = 16$); ■, locally invasive CaP ($n = 7$); *C*, prognosis: □, benign ($n = 15$); ■, unfavorable ($n = 8$); and *D*, total PSA level before surgery: □, 0.1–4.0 ng/ml ($n = 7$); ▨, 4.0–10.0 ng/ml ($n = 7$); ■, >10.0 ng/ml ($n = 9$). The data were normalized to total RNA (10 μg of total RNA were analyzed for each sample) and by setting at 100% the value obtained in the correspondent normal counterpart from the same gland. t $\text{test}_{[p.c.]}$ analysis: *, $P < 0.05$; **, $P < 0.01$.

accumulation was higher in the cancerous portion of the gland. However, this did not reach statistical significance (t $\text{test}_{[p.c.]}$). This was consistent with the data shown previously in Fig. 1*B*.

Similar results were obtained when the same data were analyzed as a function of other classification systems. For example in tumors showing capsular penetration (Fig. 3*B*), the up-regulation of *ODC* and *AdoMetDC* and the down-regulation of *Gas1* and *SGP-2* were all statistically significant. *H3* overexpression reached statistical significance in organ confined tumors, where also ODC and SSAT mRNAs levels were significantly higher than controls.

In patients with negative prognosis (*i.e.,* with at least one of the following features: regular increases of total PSA after surgery; lymph node involvement; presence of distant metastasis; Fig. 3*C*), *ODC*, *AdoMetDC*, and *SSAT* were significantly up-regulated, and *Gas1* and *SGP-2* were significantly down-regulated. However, *SSAT* was significantly overexpressed in patients with favorable prognosis as well.

When the data were analyzed as a function of total PSA levels at diagnosis (Fig. 3*D*), significant differences between cancerous and normal tissue levels of ODC, SSAT, H3, and SGP-2 transcripts were observed in patients with the highest total PSA levels (>10 ng/ml),

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whereas ODC was significantly overexpressed, even in patients with total PSA in the range of 0.1–4.0 ng/ml. Total PSA values are related mainly to tumor volume and to the disruption of the barriers between the site of PSA production and the capillary bed (20). The concurrent increase in the number of cells in S phase (as indicated by histone H3 and ODC mRNAs overexpression) and total PSA levels may be related to a higher growth rate and invasiveness of CaP.

Independently of the classification system used for CaP specimens, tumor levels of SGP-2 mRNA were ~50% lower compared with the normal counterpart. This was even observed in well-differentiated tumors, thus representing an early event during CaP progression that may result, among other molecular mechanisms, from a specific genetic loss. The *clusterin* gene is present as a single copy and maps to human chromosome 8 (21) in the 8p21 region (22). It has been hypothesized that one (or more) tumor suppressor genes are located on human chromosome 8, which contains one of the most frequently deleted loci observed in prostate cancer cells, 8p22–8p21 (23). Furthermore, when Dunning rat prostate carcinoma cells were used as recipients for human chromosomes, metastasis suppressor activities were also identified on chromosome 8 (24). The role played by potential deletion of the *clusterin* locus in the expression levels of SGP-2 is currently under investigation in our laboratory.

Statistically significant correlations between the levels of the transcripts within the same CaP specimens, grouped as shown in Table 1, were sought by applying the Pearson *r* test. Of the 28 possible correlations, 4 were found in the normal portions of the gland and three in the cancerous portions (Table 1A). When the specimens were grouped according to the differentiation grade (Table 1B), local invasiveness (Table 1C), and prognosis (Table 1D), the number of significant correlations was always lower with higher malignancy. This picture suggests that specific biochemical lesions, consisting of the progressive loss in the coordinate levels of expression of the genes studied, are major events in the metabolic derangement that accompanies neoplastic transformation.

Some of the correlations found may help in the interpretation of basic changes that accompany, at the molecular level, transformation of the prostate gland. Table 1A confirms the positive correlation between ODC/SSAT levels in normal tissue. Thus, enhancements of ODC activity and consequent increases in polyamine concentrations are usually followed by induction of SSAT, which contributes to maintenance of intracellular polyamine homeostasis. The observed overexpression of ODC mRNA in early S-phase cells and the elevation of SSAT mRNA in late S and G_2-M phases (6) support this correlation. The positive correlation between H3/SSAT mRNA levels can also be reconciled, because H3 mRNA levels peak in S phase. Also, although the ODC/SSAT correlation is statistically significant in less malignant tumors (Table 1, C and D), it is lost in more advanced cancers (Table 1, B–D). Therefore one of the mechanisms that prevents polyamine overaccumulation is lost with increasing tumor grade and loss of differentiation.

OAZ and SSAT mRNA levels were significantly correlated not only in the CaP specimens taken together (Table 1A) but also in all three groups of advanced cancers (Table 1, B–D). This implies that changes in the expression of these two genes, which are essential for the regulation of intracellular polyamine homeostasis, occur in a coordinate manner even after neoplastic conversion. It is likely that the increased expression of *OAZ* and *SSAT* in cancer cells prevents polyamine concentration to reach levels that would be toxic to the cell.

Table 1 *Correlations (Pearson r test)*

Specimen classification	No. of significant correlations (of 28)	Correlated genes	Statistical parameters
A. Overall correlations			
Normal ($n = 23$)	4	*ODC/SSAT*	$r = 0.537$; $n = 19$; $P = 0.018$
		SSAT/H3	$r = 0.531$; $n = 18$; $P = 0.023$
		AdoMetDC/GAPDH (−)	$r = -0.508$; $n = 21$; $P = 0.019$
		Gas1/GAPDH	$r = 0.501$; $n = 21$; $P = 0.021$
Cancerous ($n = 23$)	3	*ODC/H3*	$r = 0.589$; $n = 18$; $P = 0.010$
		OAZ/SSAT	$r = 0.671$; $n = 21$; $P = 0.001$
		Gas1/GAPDH	$r = 0.541$; $n = 21$; $P = 0.011$
B. Differentiation grade			
Cancerous: well differentiated ($n = 7$)	3	*H3/GAPDH*	$r = 0.955$; $n = 4$; $P = 0.043$
		GAPDH/SSAT	$r = 0.819$; $n = 7$; $P = 0.024$
		Gas1/GAPDH	$r = 0.832$; $n = 6$; $P = 0.040$
Cancerous: moderately differentiated ($n = 7$)	2	*OAZ/SGP-2* (−)	$r = -0.932$; $n = 6$; $P = 0.007$
		Gas1/SGP-2 (−)	$r = -0.912$; $n = 6$; $P = 0.011$
Cancerous: poorly differentiated ($n = 8$)	1	*OAZ/SSAT*	$r = 0.760$; $n = 8$; $P = 0.029$
C. Tumor localization			
Cancerous: organ confined ($n = 15$)	4	*H3/ODC*	$r = 0.672$; $n = 12$; $P = 0.017$
		ODC/SSAT	$r = 0.668$; $n = 12$; $P = 0.013$
		GAPDH/SSAT	$r = 0.592$; $n = 14$; $P = 0.026$
		Gas1/GAPDH	$r = 0.704$; $n = 15$; $P = 0.003$
Cancerous capsular penetration ($n = 7$)	2	*OAZ/SSAT*	$r = 0.907$; $n = 6$; $P = 0.013$
		AdoMetDC/SGP-2	$r = 0.843$; $n = 6$; $P = 0.035$
D. Prognosis			
Cancerous: benign ($n = 13$)	4	*ODC/SSAT*	$r = 0.606$; $n = 11$; $P = 0.048$
		OAZ/SSAT	$r = 0.568$; $n = 13$; $P = 0.043$
		GAPDH/SSAT	$r = 0.673$; $n = 13$; $P = 0.012$
		GAPDH/Gas1	$r = 0.708$; $n = 13$; $P = 0.007$
Cancerous: unfavourable ($n = 9$)	1	*OAZ/SSAT*	$r = 0.760$; $n = 8$; $P = 0.029$

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Interestingly, in the normal tissue contiguous to a poorly differentiated prostate cancer, a significant negative correlation ($t = -0.746$; $n = 8$; $P = 0.034$) between the decrease in *ODC* and increased *clusterin* gene expression was observed (compared with the adjacent tumor tissue). This is a typical response that we have shown to be induced during tissue involution and/or decreased rate of cell proliferation (25, 26) and is probably attributable to local cytotoxicity caused by growth of the tumor.

By means of the logistic regression analysis, we next sought to use the changes in gene expression observed in the cancer tissues relative to matched normal prostate to develop an alternative classification system for prostate tumors. This was compiled using the data from the 18 CaPs for which quantification of all of the transcripts had been carried out (Table 2).

In Table 2A the classification of patients was based only on the expression of the markers of tissue proliferation and cell quiescence, H3 and Gas1, respectively. Using this method, classification of 61.1% of the patients coincided with the differentiation (Gleason) grade. When the same patients were classified according to specific markers of the S phase, H3 and ODC, this percentage increased to 66.7% (Table 2B), which is probably related to higher proliferation rate in higher Gleason grade prostate cancers. Finally, when all of the genes studied were used to produce the classification, the overall prediction in 72.2% of the patients coincided with the differentiation grade (Table 2C). When used to discriminate between organ confined or locally invasive CaP, the levels of *H3* and *Gas1* gene expression were able to predict tumor localization in 72.2% of the patients (Table 3A), including all of the genes in the analysis brought the overall prediction to 83.3% (organ confined, 75%; capsular penetration 100%; Table 3B). Thus, the molecular classification was generally in good agreement with both the Gleason grade and local invasiveness.

Closer analysis of the outliers from the correlation of molecular and clinical classifications revealed a nonrandom distribution. All of the patients not correctly classified according to the differentiation grade and tumor localization (Tables 2C and 3B) were predicted as belonging to the nearest group. Intriguingly, in two patients with moderately differentiated CaPs, according to the Gleason grading, but classified in the poorly differentiated group by the molecular classification (using the complete gene set), the total PSA after radical prostatectomy has now begun to rise, in a 1-year follow-up period, to 2.0 and 0.5 ng/ml, respectively, suggesting the possibility of tumor relapse. Conversely, in the two poorly differentiated tumors that were ascribed by the molecular classification to the moderately differentiated group, the total PSA after radical prostatectomy remained constantly <0.1 ng/ml (Table 2C), indicative of successful treatment. The molecular classification system correctly predicted all of the tumors showing capsular penetration, when all of the genes were included in the analysis (Table 3B). Three patients classified as belonging to the organ-confined group were ascribed to the more aggressive phenotype on the basis of gene expression. For two of them, the probability of being included in the capsular penetration group was 56 and 51%, respectively. However for the third patient, whose probability of being included in the capsular penetration group was 86%, the total PSA levels determined after surgery were constantly increasing, and tumor relapse has now been demonstrated.

Table 2 *CaP classification by logistic regression analysis: Differentiation grade*

Observed	Predicted[a] W	M	P	Correct (%)
A. Genes included in the analysis: *H3* and *Gas1*				
W	3	0	1	75.0
M	1	2	3	33.3
P	1	1	6	75.0
Correctly classified (overall)				61.1
B. Genes included in the analysis: *H3* and *ODC*				
W	3	0	1	75.0
M	1	4	1	66.7
P	2	1	5	62.5
Correctly classified (overall)				66.7
C. All of the genes are included in the analysis				
W	3	1	0	75.0
M	0	4	2	66.7
P	0	2	6	75.0
Correctly classified (overall)				72.2

[a] W, well differentiated; M, moderately differentiated; P, poorly differentiated.

Table 3 *CaP classification by logistic regression analysis: Tumor localization*

Observed	Predicted[a] O.C.	C.P.	Correct (%)
A. Genes included in the analysis: *H3* and *Gas1*			
O.C.	9	3	75.0
C.P.	2	4	66.7
Correctly classified (overall)			72.2
B. All of the genes are included in the analysis			
O.C.	9	3	75.0
C.P.	0	6	100.0
Correctly classified (overall)			83.3

[a] O.C., organ confined; C.P., capsular penetration.

These good correlations, even in a heterogeneous tumor like CaP, indicate that changes in expression of all of the genes studied in the CaP specimens are involved in tumor progression. Although neoplastic transformation is often the consequence of aberrant expression of oncogenes and tumor suppressor genes, it is also accompanied by disruption of the coordinate expression of set of genes controlling the levels of specific metabolites. In a tissue-specific manner, it is likely that more precise means for the classifications of tumor malignancy may result from the definition of expression profiles of these specific metabolically related sets of genes.

Longer patient follow-up should provide the information that will enable us to understand whether the particular features shown by this new taxonomic tool will be useful to provide new perspectives about CaP progression that cannot be obtained by the traditional systems and to augment the current available clinical tools.

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Tumor Progression Is Accompanied by Significant Changes in the Levels of Expression of Polyamine Metabolism Regulatory Genes and Clusterin (Sulfated Glycoprotein 2) in Human Prostate Cancer Specimens

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