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THE EXPRESSION OF METALLOTHIONEINS ON IMPRINT SMEARS OF PROSTATE CARCINOMA: CORRELATION WITH CLINICOPATHOLOGIC PARAMETERS AND TUMOR PROLIFERATIVE CAPACITY

Paulina Athanassiadou[1], Athanasios Bantis[2], Maria Gonidi[1], Peter Athanassiades[1], Eleni Agelonidou[1], Dimitra Grapsa[1], Polyxeni Nikolopoulou[1], and Efstratios Patsouris[1]

[1]Pathology Laboratory-Cytology Unit, Medical School, Athens University, Athens; [2]Urology Department, University Hospital, Alexandroupolis, Greece

Aims and background: **Metallothioneins are a family of metal-binding cysteine-rich proteins that play an important role in cellular processes such as proliferation and apoptosis, protection against oxidative stress and metal ion homeostasis and detoxification. Recent findings suggest that metallothioneins might play a significant role in the development and progression of prostate cancer. It has been also demonstrated that Ki-67 expression may have prognostic value for disease-free survival in cases of prostate carcinoma.**
Study design: **Imprint smears samples obtained from 70 patients immediately after radical prostatectomy for prostatic carcinoma were immunostained with monoclonal antibodies against metallothioneins and Ki-67. Metallothionein expression was correlated with Ki-67 immunostaining, Gleason score, stage, preoperative prostate-specific antigen levels and biochemical recurrence.**
Results: **Metallothionein expression was shown to correlate strongly with Gleason score (P <0.001) and significantly with pathological staging and Ki-67 immunostaining (P <0.001, P <0.05, respectively). In contrast, no significant association between metallothioneins and preoperative PSA was demonstrated. Both of the studied markers (metallothioneins and Ki-67) correlated with recurrence (P = 0.009, P = 0.006, respectively).**
Conclusions: **The present findings support the independent predictive value of metallothioneins and Ki-67 in prostate cancer. However, additional data are needed in order to reveal the factors that influence the expression of metallothioneins in epithelial neoplastic cells and clarify their mechanism of action.**

Key words: immunocytochemistry, Ki-67, metallothioneins, prognostic factors, prostate cancer.

Introduction

Several studies suggest a role for metallothioneins (MTs) in cancer development, treatment resistance and prognosis[1,2]. Furthermore, immunohistochemical MT expression in various tumors has been associated either with processes related to carcinogenesis or with chemoresistance and radiotherapy resistance[1,3]. MTs are low-molecular-weight, metal-binding, cysteine-rich, intracellular proteins that play an important role in cellular processes such as proliferation and apoptosis, protection against oxidative stress and metal ion homeostasis and detoxification[4,5]. In humans, functional MT isoforms are encoded by a family of genes located at chromosome 16q13[6-10]. The induction of different MT isoforms has been shown to be dependent on cell type and to be specifically regulated[11,12]. Recently, Abdel-Magged and Agrawal[13] showed that down-regulation of MT-2A induces growth arrest and apoptosis in human breast carcinoma cells, suggesting the involvement of the MT-2 isoform in the proliferative activity of cancer cells.

Recent findings suggest that MTs might play a significant role in the development and progression of prostate cancer[14-16]. Zinc is involved in several physiologic processes, including cell growth and proliferation. In normal prostate cells, zinc levels are high, but there is a marked decrease in prostate cancer[17]. Oral cadmium exposure induce a dose-related induction of proliferative lesions as tumors and atypical hyperplasia in the prostate. Zinc can have an important impact on cadmium-induced carcinogenesis. Excess zinc appears to facilitate cadmium-induced prostate cancer by preventing testicular toxicity and maintaining testosterone production[18].

The Ki-67 antigen is associated with cell proliferation and detects tumor proliferative capacity. Furthermore, Ki-67 expression may have prognostic value for disease-free survival in cases of prostate carcinoma[19-24].

The aim of this study was to investigate the expression of MTs (MT-1 and MT-2 isoforms) in prostate cancer cells and to correlate the results with Ki-67 expression and clinicopathologic parameters.

Materials and methods

Samples were obtained from 70 patients who underwent radical prostatectomy for prostate cancer. Patient age ranged from 59 to 75 years (mean, 67.11). Imprint smears were taken from different areas of macroscopically estimated prostate cancer, immediately after excision of the prostate. After air drying, cytologic smears were fixed in ethanol/acetone 1:1 for 10 min and stored at -70 °C until used. The histopathological diagnoses

Correspondence to: Pauline Athanassiadou, MD, MIAC, Associate Professor, Pathology Laboratory-Cytology Unit, Medical School, Athens University, 75 Mikras Asias street, GR-115 27 Athens, Greece. Tel +30-210-7462171; fax +30-210-7462157; e-mail pathanas@cc.uoa.gr

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were performed using sections from the same samples that were used for the imprints. The TNM system (based on the staging system of the American Joint Committee on Cancer)[25] was used for pathological staging, and grading of the primary cancer was evaluated according to the Gleason score system. None of the patients had been treated with radiation or androgen depletion prior to prostatectomy.

Follow-up after surgery ranged from 6 (early biochemical recurrence) to 60 months (longest time without biochemical recurrence) (mean, 54) and included evaluation of serum prostate-specific antigen (PSA) at regular clinical intervals. PSA values greater than 0.2 ng/ml on successive measurements was defined as relapse. The clinicopathological data of prostate cancer cases are reported in Table 1.

Immunocytochemical staining for MTs and Ki-67 was performed by the avidin-biotin complex-immunoperoxidase method as previously reported[26]. After rinsing the smears with 0.01 M phosphate-buffered saline (PBS), normal horse serum was applied for 20 min to block nonspecific antibody binding. Subsequently, smears were incubated overnight at 4°C with the primary antibodies. After additional rinsing in PBS, smears were incubated with rabbit anti-mouse biotinylated immunoglobulins (Vector Laboratories, Bur-lingame, CA, USA) diluted 1:200 for 30 min at room temperature and then incubated with avidin-biotinylated peroxidase complex (Vectastain ABC Kit, Vector Laboratories) for 30 min. The peroxidase reaction was developed with a 0.5 mg/ml solution of 3.3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co. St. Louis, MO, USA) supplemented with 0.01% H_2O_2. Finally, smears were counterstained with Mayer's hematoxylin.

Table 1 - Clinical characteristics of 70 patients treated with radical prostatectomy

	Data of patients	
	No.	(%)
Age (years)		
<65	14	20.0
65-69	35	50.0
>70	21	30.0
Stage		
T2a	44	62,9
T2b	17	24.3
T2c	7	10.0
T3a	2	2.9
Gleason score		
2-4	19	27.1
5-6	30	42.9
≥7	21	30.0
Pretreatment PSA levels (ng/ml)		
0-5	13	18.6
5.1-9.9	36	51.4
≥10	21	30.0

T2a, tumor involves 50% of a lobe or less; T2b, tumor involves more than 50% of a lobe; T2c, tumor involves both lobes; T3a, unilateral extracapsular extension of the tumor

A monoclonal antibody against the Ki-67 (Dakopatts, Glostrup, Denmark) was used at a dilution of 1:40, and also a specific monoclonal mouse antibody of MT clone E9 (Neo Markers, Fremont, CA, USA) was used at a dilution of 1:600.

For MTs and Ki-67, immunolabeling negative controls were included by omitting the primary antibody and positive controls inserted by including a known positive with each batch.

In comparison to fresh imprint smears, tissue sections present a lot of difficulties with regard to estimation of Ki-67 and MT immunoreactivity. Depending on the thickness of the section, there will always be a number of cells that are either sliced or overlapped, the first leading to false low and the latter to false high immunoreactivity[27].

Ki-67 immunostaining was considered positive when the nuclei of tumor cells were stained brown and when a partial diffuse and granular brown pattern was shown (Figure 1). For statistical analysis, the results of Ki-67 positivity were typically expressed on a four point score as follows: staining of <20% of cells (-), 21-40% (+), 41-60% (++), 61-100% (+++). Nuclear Ki-67 staining was positive if >21% and negative if <20% of cells stained.

MT immunoreactivity was observed both as nuclear and cytoplasmic staining (Figure 2) and expressed as intensity percentage score (IPS), where IPS = staining intensity X percentage of immunopositive cells. Staining

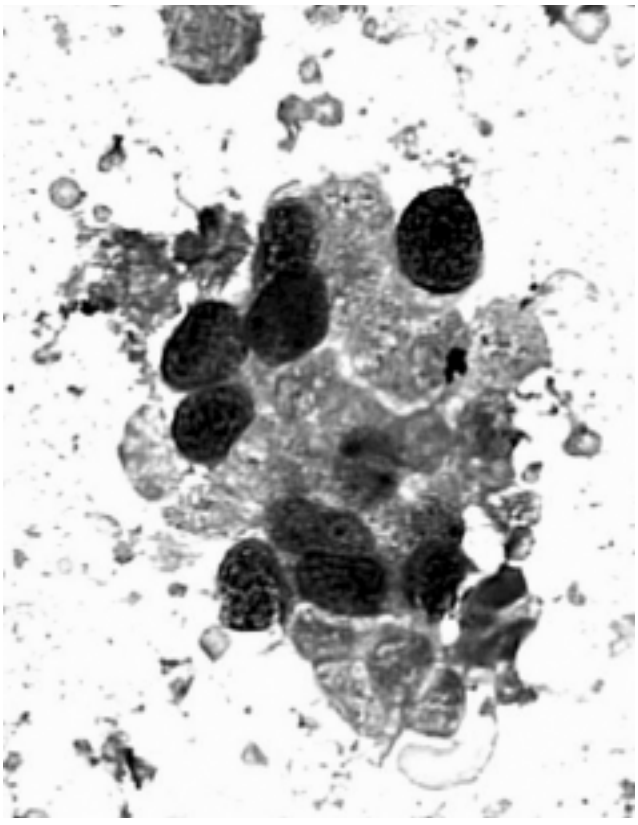

Figure 1 - Clusters of malignant cells from a poorly differentiated prostate adenocarcinoma, with positive nuclear immunoreaction for Ki-67 (×500).

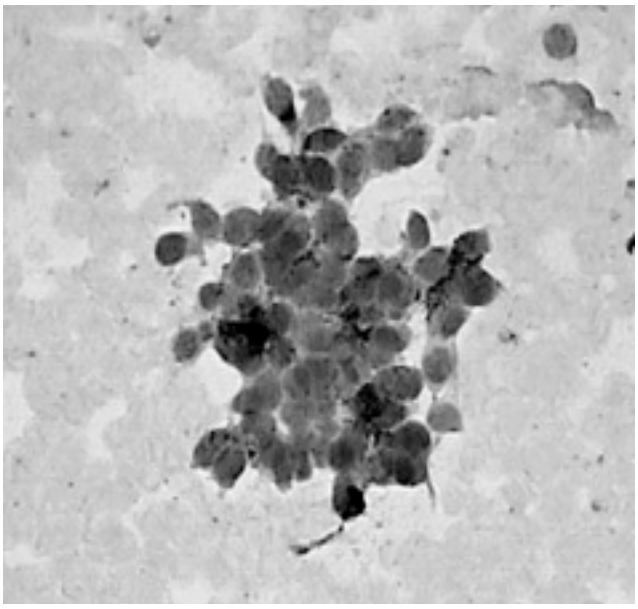

Figure 2 - Sheets of prostate adenocarcinoma cells, poorly differentiated, with positive nuclear immunoreaction for metallothioneins.

intensity was designated as weak (+), moderate (++) and strong (+++). The percentage of MT-positive cells was counted under a light microscope, and a total of 10 high power fields were randomly chosen. In cases where staining was heterogeneous in the slide, examined fields included those with the highest and those with the lowest percentage of stained cells.

The relationship of MTs with all prognostic parameters (preoperative PSA serum values, Gleason score, stage, and Ki-67 expression) was assessed by one-way analysis of variance (ANOVA) followed by tests of multiple comparisons, since MTs did not deviate from normality (Kolmogorov-Smirnof test, $P = 0.227$). The simultaneous effect of all variables on MT expression was investigated by multiple linear regression. Disease-free survival was assessed by Cox's proportional hazard regression model.

Results

MT protein expression was detected in 44 (62.86%) of the prostate carcinoma smears (Figure 3). The percentage of MT- (MT1-MT2 isoforms) positive cells ranged from 8-92%, with a mean of 59.7 ± 3.1% (± SEM), and the IPS ranged from 5 to 215%, with a mean of 94.3 ± 5.0.

Immunocytochemical expression of the cell proliferation-associated protein Ki-67 is shown in Figure 3. Positive expression for Ki-67 was detected in 63 (90%) of the prostate carcinoma smears.

Table 2 shows the significant correlation between the degree of Gleason score and MTs ($P < 0.0001$) and Ki-67 ($P < 0.001$) expression. We observed that an increasing Gleason tumor grade was associated with an increase in the percentage of cells stained positively for Ki-67 and MTs.

Overexpression of MTs was not associated with increased pretreatment PSA serum levels (4 ng/ml) ($P = 0.8184$), in contrast with Ki-67 expression ($P = 0.05$) (Table 2).

The distribution of MTs and Ki-67 expression in prostate carcinomas according to histopathological staging is shown in Figure 4. There was a statistically significant association in MTs ($P = 0.002$) and Ki-67 ($P =$

Table 2 - Distribution of ki-67 and MTs expression in prostate carcinomas according to Gleason score and preoperative PSA values (ng/ml)

			No. MTs		No. Ki-67	
Gleason score	No	%	(+) %	(-) %	(+) %	(-) %
2-4	19	27.1	26	74	38	62
5-6	30	42.9	32	68	33	67
≥7	21	30.0	48	52	54	46
		P	<0.0001		<0.001	
PSA ng/ml						
0-5	13	18.6	26	74	30	70
5.1-9.9	36	51.4	37	63	67	33
≥10	21	30.0	47	53	85	15
Total	70	100				
		P	0.8184		0.05	

Ki-67 (+): >21% of cells stained; MTs (+): moderate and strong staining intensity.

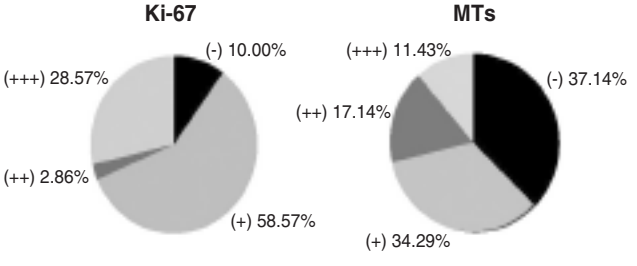

Figure 3 - Percentages of immunostaining intensity (+,++,+++) of Ki-67 and metallothioneins in all prostate adenocarcinoma smears studied.

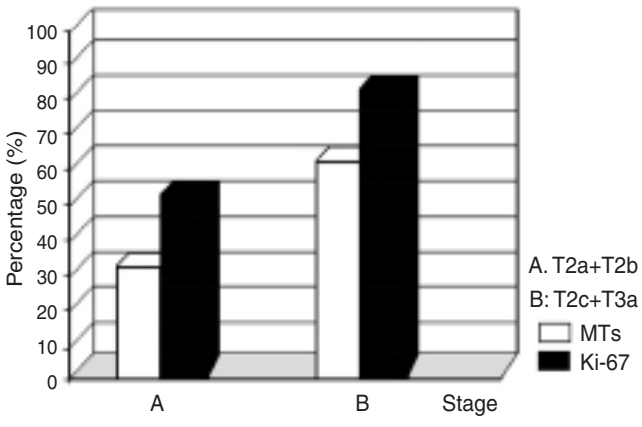

Figure 4 - Distribution of metallothioneins and Ki-67 expression in prostate carcinoma smears according to histopathological staging.

0.004) immunoexpression values when histopathological staging was considered. High overall MT and Ki-67 expression was consistently associated with increased stage (T2c+T3a) compared to the low MT and Ki-67 expression (T2a, T2b stage).

Overexpression of Ki-67 in prostate carcinoma cell smears was associated with an increase in the positive expression of MTs ($P = 0.05$).

The logrank test and Cox's model demonstrated that MT and Ki-67 expression had significant prognostic value ($P = 0.009$ and $P = 0.007$, respectively) for disease-free survival (Tables 3 and 4). A higher recurrence was noted for those patients who had high MT and Ki-67 expression compared with those who had low expression. Of the 70 patients with analyzed specimens, 10 (14.3%) had a biochemical recurrence of the disease during the follow-up period (mean, 37 months).

Table 3 - Assessed by multiple linear regression model (Dependent variables: MTs and Ki-67, long rank test)

	Log-rank test						95% CI for HR	
	B	SE	Wald	df	Sign	HR	Lower	Upper
MTs	.132	.060	5,773	1	.009	1.863	1.789	1.944
Ki-67	.145	.066	6,371	1	.007	1.115	1.060	1.170

Table 4 - Cox's model variable not in the equation

Cox's model: variable not in the Equation	Score	df	Sign
Gleason score	.714	1	.342
Ki-67	.886	1	.523
MTs	.067	1	.710
PSA	.114	1	.683
Stage	.216	1	.642

Residual Chi Square = 14,732 with 6df Sign = .054

Discussion

Published data concerning the role of MTs in prostate cancer are limited. According to the reported results, MT expression is highly variable among prostate carcinomas[15,16]. Moreover, an increased MT expression has been demonstrated in prostate tumors in comparison with normal tissue, and MT immunopositivity has been strongly correlated with tumor grade[14-16]. These findings provide evidence that MTs might be implicated in prostate carcinogenesis and also serve as a marker. The role of MTs in other forms of cancer has been previously investigated in several studies. Increased expression of MTs has been observed in carcinomas of the breast, colon, ovary, urinary bladder and various other tumors, and it has been suggested that MTs may play a role in regulating cell proliferation and apoptosis[4,5,14,28]. In breast cancer, MT overexpression has been shown to predict a worse survival and correlated with increased proliferation – as indicated by Ki-67 immunopositivity – whereas similar results were observed in colorectal, urinary bladder and ovarian carcinomas[5,6,14,29-31]. MT expression has been also linked to reduced apoptosis in hepatocellular and nasopharyngeal carcinomas[5,32,33].

In the present study, positive MT expression was detected in 62.86% of prostate cancer smears. In order to investigate the role of MTs as candidate tumor markers in prostate cancer, we correlated their expression with prognostic factors such as pathologic staging, Gleason score, preoperative PSA serum levels and Ki-67. We found a strong correlation between MT expression and Gleason score, whereas no significant association with PSA was demonstrated. Similar results were observed by other investigators, as in all previous studies MT expression was found to correlate strongly with Gleason score[15,16]. The published data, to our knowledge, concerning the correlation of MTs with preoperative PSA failed to demonstrate any significant association between MTs and PSA[15]. The correlation of MTs with preoperative PSA is of great importance for determining their prognostic significance, since PSA currently is the most important marker for the early diagnosis of prostate cancer and its biochemical recurrence after surgical therapy.

The association between MT expression and pathological staging was found to be significant in our study, in contrast with previous studies [15,16], which may suggest its prognostic value in prostate carcinoma. MTs expression has been previously associated with positive Ki-67 staining in breast tumors, colon tumors and other cancers, such as nasopharyngeal cancer[14,34,35]. In our study, Ki-67 immunostaining in prostate cancer smears ranged from 12-97%, with a mean of 90%. Furthermore, a significant correlation was observed between MT protein expression and Ki-67 ($P = 0.05$). According to other studies which are in agreement with our results, there are two possible mechanisms by which MTs could influence cell proliferation[8]. MTs could supply zinc ions to enzymes such as DNA and RNA polymerases, which are critical in cell replication processes[36]. There is also experimental evidence that zinc ions are transferred between MTs and zinc proteins[37,38]. Normal prostate glands contain higher levels of zinc than cancerous tissues[39]. Hasumi *et al.*[40] demonstrated that MT concentration in the peripheral zone of human prostate was significantly higher than in prostate cancer tissues.

Another possibility is that MTs directly interact with transcription factors involved in the intricate signaling mechanisms that stimulate cell proliferation[8].

The increasing expression of MTs in advanced grade and stage prostate cancer and the inherent chemoresistance (recurrence) in prostate cancer suggest a strong association between MTs and chemoresistance in prostate cancer. MT expression is up-regulated by androgens, which suggests a complex interaction between androgen responsiveness and chemoresistance in prostate cancer[41]. Literature data strongly support constitutive expression of MTs in the prostate gland and an altered expression with malignant transformation and cancer progression and induction of chemoresistance in prostate cells[42].

With regard to the disease-free survival after radical

prostatectomy, the two studied markers correlated with recurrence by the Cox proportional hazard regression model. The significant co-expression of MTs and the proliferation-associated protein Ki-67 in our study supports their collective role in tumor proliferation and that they are independent predictors of disease recurrence after radical prostatectomy. This is not surprising, given that these markers in the 10 patients with recurrence were correlated with high tumor grade and poor differentiation, possibly reflecting a population of actively dividing cells.

In conclusion, our results suggest that MT expression is positively correlated with Gleason score, staging and Ki-67 but not with PSA. However, we believe that further research is needed in order to clarify the prognostic significance of MTs and their isoforms in prostate cancer as well as the molecular mechanisms underlying the role of MTs in prostate oncogenesis. Even though we are still far from establishing the role of MTs as a prognostic marker in cancer, we cannot deny that these molecules deserve our close attention.

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