Increased Expression of High Mobility Group Protein I(Y) in High Grade Prostatic Cancer Determined by in Situ Hybridization

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ABSTRACT

In a previous study using the Dunning rat prostate cancer model, we found high mobility group protein I(Y) (HMG-I(Y)) to be overexpressed in metastatic tumor lines when compared to nonmetastatic lines. Hence, overexpression of this 12-kDa non-histone chromosomal protein may be associated with tumor progression. Firstly, by Northern analysis we showed that HMG-I(Y) expression increases in high grade prostate tumors. These studies, however, required fresh material, and clinical follow-up was limited. To overcome this problem paraffin-embedded material must be made amenable for determination of HMG-I(Y) expression in retrospective studies. RNA in situ hybridization enables the evaluation of mRNA levels in such material. We studied tumors from 71 patients with prostate cancer. The microscopic analysis of each sample included: (a) hybridization on sections with sense HMG-I(Y) and (b) 28S rRNA probes (nonspecific signal); (c) hybridization with antisense 28S rRNA (RNA preservation); (d) hybridization with an antisense HMG-I(Y) probe [quantification of HMG-I(Y) mRNA in the expressing areas]. Data were quantified using an image analysis system. High expression of HMG-I(Y) was observed in regions with high Gleason grade (4 and 5); whereas in lesions of Gleason grade 3, both weak and no expression was observed. In areas of grade 1 and 2, as well as in normal glands, low or no expression was found. We conclude that HMG-I(Y) expression assessed by RNA in situ hybridization is related to tumor differentiation in prostate cancer. These findings indicate that HMG-I(Y) expression may be a marker in prostate cancer diagnosis, and the possible clinical implication of expression of this gene in malignancy is discussed in this report.

INTRODUCTION

Prostate cancer is now the second leading cause of cancer-related deaths in United States males. In men older than 50 years, 1 in 11 will be clinically diagnosed with prostate cancer. More than one-third of these patients will die from their disease (1). In these patients the tumor can remain stable for years or clinically progress rapidly towards a life-threatening situation. Prognostic indicators for the diverse biological tumor behavior are thus urgently needed.

Investigations regarding the activation and/or inactivation of oncogenes (2-4), growth factors, and tumor suppressor genes (5-8) have not yet revealed consistent differences between the latent and aggressive prostate cancers (9). Ha-ras (2) and p53 (7) mutations, as well as loss of Rb expression (5), are found to be not a frequent step in the molecular cascade leading to prostate cancer progression. We have therefore tried to identify suitable progression markers and genetic changes associated with prostate cancer progression. One of the interesting findings from these investigations was the loss of E-cadherin function, which is associated with the progression of prostate cancer (10). Another potential progression marker was identified upon differential screening of metastatic versus nonmetastatic Dunning sublines (i.e., MAT-Ly-Lu versus H tumor). Three clones showed overexpression in the metastatic rat prostatic tumors and the nucleotide sequence analysis indicated that one of the clones was identical to the HMG-I(Y)3 (11). High mobility group proteins I and Y are two isoforms of a non-histone nuclear DNA-binding protein and result from alternative splicing from a single functional gene (12). They are characterized by: a low molecular weight; high content of basic and acidic amino acids; and specific binding to the minor groove of A:T-rich sequences (13, 14) presumably in a way similar to that of antitumor and antiviral drugs (Netropsin, Distamicin) and the dye Hoechst 33258 (15, 16). They can also become phosphorylated by cdc2 kinase (17), which presumably modulates DNA-binding activity. It has been also shown that they are involved in the condensation of chromosomes during metaphase and in the maintenance of the undifferentiated state of chromatin (18-22). It is of particular interest that HMG-I(Y) overexpression was also found to be up-regulated in de-differentiated myeloid cells (23) and during neoplastic transformation (11, 18, 24, 25). In order to investigate the possibility of using HMG-I(Y) as a progression marker we anticipated immunohistochemical analysis. However, probably due to the low immunogenicity of the protein, as yet no routinely applicable antibody and protocols are available. We therefore studied HMG-I(Y) expression in 71 prostate cancer specimens by RISH. Image analysis techniques were applied for quantitation of RNA expression, and the in situ hybridization results were compared with Gleason grade and stage.

MATERIALS AND METHODS

Specimens were obtained by transurethral resection of the prostate, lymphadenectomy, or radical prostatectomy. They were either snap frozen immediately after surgery or fixed in formalin and embedded in paraffin.

Preparation of Frozen Sections

Serial sections from the frozen tissues were cut on a cryostat at 8 µm, mounted on poly-l-lysine-coated slides, covered with Carbowax fixative (2% polyethylene glycol 1500, in 50% ethanol), and heated in a microwave oven for 30 s. For the microwave fixation method, we used a household microwave oven with a maximum power of 750 W and a frequency of 2450 MHz (26, 27). The sections were rinsed twice for 5 min in PBS, washed for 5 min in glycine/PBS, and incubated in 0.3% Triton X-100/PBS for 10 min. After rinsing for 1 min in PBS, sections were postfixed in 4% paraformaldehyde/PBS for 5 min, rinsed in PBS, and acetylated in freshly prepared 0.25% acetic anhydride/0.1 M triethanolamine, pH 8, for 10 min (28). The slides were then finally dehydrated in gradually increasing concentrations of ethanol prior to hybridization.

Preparation of Paraffin-embedded Material

Four-µm sections were mounted on acid-precleared poly-l-lysine-coated microscope slides and heated overnight at 50°C. The sections were deparaffinized, rehydrated in PBS, and treated with proteinase K (10 µg/ml) in 20 mM

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3 The abbreviations used are: HMG-I(Y), high mobility group protein I(Y); RISH, RNA in situ hybridization; PBS, phosphate-buffered saline (1× PBS = 137 mM NaCl-2.7 mM KCl-8.1 mM Na2HPO4·1.5 mM KH2PO4, pH 7); SSC, standard saline-citrate (1× SSC = 300 mM NaCl-30 mM sodium citrate).
Tris·HCl, pH 7.5–5 µm EDTA for 15 min at 37°C before a postfixation by Dr. G. J. M. Pruyn and Dr. P. C. Van der Vliet (University of Utrecht, Utrecht, the Netherlands). The plasmid was digested with EcoRV to generate a template for antisense transcription. Alternatively, digestion with BamHI resulted in a template for sense probe synthesis, used as a negative control. To test the preservation of RNA, we used a 28S rRNA-specific probe cloned in pGem-II (Promega). [35S]UTP-labeled single-stranded RNA probes were prepared according to the method of Melton et al. (29, 30), degraded to an average length of 200 nucleotides by alkaline hydrolysis (31), precipitated in ethanol, and resuspended in 100 µl 10 µm Tris·Cl, pH 7.5–1 µm EDTA. The specific activity of the radiolabeled probes, aliquoted and stored at −80°C until use, was approximately 1 × 107 cpm/µg template DNA. Twenty-five µl of the hybridization buffer [50% deionized formamide-2× SSC-10% dextran sulfate-5× Denhardt’s (50× Denhardt’s = 5 g Ficoll 400, 5 g polyvinyl polydriodine, and 5 g bovine serum albumin in 500 ml H2O)-10 µm dithiothreitol, and 1 mg/ml tRNA] was mixed with 2 × 106 cpm of [35S]UTP-labeled probes and applied to the sections. After the sections were covered with coverslips, overnight hybridization was performed at 46°C. Coverslips were removed in 2× SSC at room temperature, washed at 46°C in decreasing concentrations of SSC (twice for 30 min 2× SSC, twice for 30 min 1× SSC, twice for 30 min 0.5× SSC). In order to reduce background due to nonspecific binding, 1 µl dithiothreitol, 1% thiosulfate, and 14 µm mercaptoethanol was added to the washing solutions. The sections were finally dehydrated, dried, and dipped in LM-1 film emulsion (Amer sham UK) for high resolution microautoradiography. After an exposure time of 5 days in tightly closed boxes at 4°C the microautoradiographs were developed in Kodak D-19 developer (4 min), washed in water (20 s), fixed in 24% thiosulfate (w/v, 4 min), rinsed in water, counterstained briefly (1 min) in hematoxylin and mounted in Permount medium before microscopic examination.

**Preservation of RNA**

In order to judge RNA preservation, samples were hybridized with sense and antisense 28S RNA probes. Samples with poorly or no preserved RNA were rejected from the analysis; i.e., the number of grains after hybridization with rRNA sense probe had to exceed 10× background.

**Quantitation by Image Analysis**

The image analysis system consisted of a video camera (MXR, HCS, Eindhoven), mounted on a routine light microscope, and a personal computer (Compaq Deskpro 386s; Compaq, Houston, TX) equipped with a framegrabber board (VFG Visionplus-AT; Imaging Technology, Inc., Bedford, MA). The output image was presented on a video monitor (PVM 1442QM; Sony, Tokyo, Japan). Software was written in TIM-image analysis language (TEA, Dordrecht, the Netherlands) and consisted of the following elements. For each tumor area 10 images were recorded at X40. A Laplace filter was applied for grain identification. The mean number of grains per image was calculated for each slide. From these data the following score for in situ staining could be derived.

\[
\text{Score A} = \frac{mRNA_{\text{HMG-I(Y)}}}{mRNA_{\text{HMG-I(Y)-}}} 
\]

This calculates the expression of HMG-I(Y+) mRNA minus its negative control HMG-I(Y−).

**Statistical Analysis**

For a comparison of the means corresponding to four groups (Benign, Gleason grade: 1–2, 3, and 4–5), the analysis of variance (F test) was performed on the A score (see "Quantitation by Image Analysis").

**RESULTS**

In order to test the probes and protocols we used a prostate cancer cell line (PC3) expressing high levels of HMG-I(Y) and analyzed this by RISH for HMG-I(Y) expression. We found high levels of HMG-I(Y) in these cells with an almost negligible background signal (data not shown). In order to evaluate HMG-I(Y) expression in human prostate cancer samples we used material harvested and stored under optimal conditions for RNA preservation (i.e., from our tissue bench, snap frozen specimens that were stored in liquid nitrogen).

**Expression of HMG-I(Y) Determined by RISH in Frozen Sections.** On basis of rRNA hybridization (rRNA+) 88% of the frozen samples showed preserved RNA and were investigated for the presence of HMG-I(Y) mRNA by RISH. Table 1 summarizes the HMG-I(Y) expression illustrated as the mean ± SD of Score A values for the nonmalignant (Score B), Gleason grade 1–2, 3, and 4–5 lesions, respectively. In the nonmalignant specimens that were tested the signals obtained after RISH did not exceed that of the background (Fig. 1, A–D). We concluded that under these conditions HMG-I(Y) expression was below the detection limit of this technique.

**Gleason Grade 1–2 Tumors.** Three of five Gleason grade 1–2 samples studied showed a signal just above the detection limit. This weak signal was specifically located in the tumor cells within the glands and the mean expression level was 206 ± 142.

**Gleason Grade 3 Tumors.** This heterogeneous group comprised small, medium, and large glands, as well as single acini with moderate variation in size and shape and cribriform growth (32). A clear expression of HMG-I(Y) was found in all tumor areas as illustrated in Fig. 1, E–H. Eighteen samples were analyzed and HMG-I(Y) expression was 1142 ± 739.

**Gleason Grade 4–5 and Anaplastic Tumors.** In Gleason grade 4–5 tumors HMG-I(Y) RISH resulted in higher "grain density" when compared to Gleason grade 1–2 and Gleason grade 3 tumors (Fig. 1, I–L). The mean Score A value for this group was 2249 ± 729.

Of 28 informative, poorly differentiated tumors (Gleason grade 4–5) with preserved RNA, HMG-I(Y) expression was higher in all but two samples where the signal was absent. Table 2 summarizes the statistical analysis of these data, and significance was found for differences in expression levels between all groups (see t and P values in Table 2). These results indicate that HMG-I(Y) expression increases with grade (see Fig. 2).

It would be extremely interesting to see how these results correlate with clinical follow-up. Unfortunately, for prostate cancer the time required for such analysis is at least 10 years hence, retrospective studies are required and the RISH technology should be applicable to archival, paraffin-embedded material.

**Expression of HMG-I(Y) Determined by RISH in Archival Material.** We therefore used RISH on archival formalin-fixed paraffin-embedded material. Among the samples taken for this purpose, 70% revealed RNA preservation and were analyzed for HMG-I(Y) expression by RISH. Table 3 summarizes the expression level for archival material samples, according to their Score A values calculated by image analysis. As in case of frozen material, with nonmalignant prostatic paraffin-embedded tissue, no HMG-I(Y) expression was found (Fig. 1C).

**Gleason Grade 1–2 Tumors.** The weak signal observed in these tumors is, as in the frozen sections, located in the tumor cells. Three
Fig. 1. A−L, representative examples of RISH on a nonmalignant tissue (A−D), and malignant specimens of Gleason grade 3 (E−H), and 4−5 (I−L). Frozen sections were hybridized with antisense rRNA (A, E, J), and sense rRNA (B, F, J) (to assess RNA preservation). The hybridization with antisense HMG-I(Y) (C, G, K), and the sense HMG-I(Y) (D, H, L) to evaluate HMG-I(Y) expression.
To gain further insight in the potential use of this marker we measured HMG-I(Y) expression in human cancers using RNA in situ hybridization, and the resulting microautoradiographic results were quantified by image analysis.

In the nonmalignant specimens no detectable expression of HMG-I(Y) was demonstrable. Interestingly in prostate cancer HMG-I(Y) expression, confined to the malignant prostatic structures, could be detected. Moreover, the level of expression correlated with the Gleason grade. This was found to be statistically significant. These results corroborate the hypothesis that HMG-I(Y) expression is related to differentiation. A particularly important aspect of this study is that not only fresh frozen tissue can be analyzed. In addition to the snap frozen tumor specimens, in which the minimum of mRNA degradation is to be expected, we also analyzed archival material. Similar results were obtained for paraffin-embedded material. The slightly lower signal observed with the latter is probably due to the differences in the procedure prior to hybridization. Indeed, formalin-fixed paraffin-embedded material required a rough pretreatment (i.e., proteinase K > 20 µg/ml) to make the mRNA accessible to the probe. During this step partial loss of mRNA may occur. We observed that, in addition to tissue damage, increasing the proteinase K concentration from 20 µg/ml to 100 µg/ml resulted in a substantial decrease of the signal (data not shown) and an alteration of tissue morphology. Similar findings reported recently, signal reduction by RNase A pretreatment (33) and a replacement of the protease treatment by saponin, have been suggested to prevent such damage and mRNA loss (34).

Having found that HMG-I(Y) expression levels correlated with tumor grade (see Fig. 1), it would be of even greater importance to evaluate the relation with the stage. In the group analyzed (53 patients) preliminary observations indicate that HMG-I(Y) expression over a score of 1300 was associated with high stage, i.e., 40% in T1 and T2 versus 70% in T3 and T4 (see Table 4). In 6 of 23 (T3) and 5 of 13 (T4) cases, the HMG-I(Y) expression level was below 1300 despite their high tumor stage. However, these exceptional cases (11 cases) had undergone hormone therapy prior to surgery which presumably interferes with the results. Thus, these preliminary analyses of HMG-I(Y) expression and tumor stage indicate that HMG-I(Y) has potential usefulness as a progression marker, albeit that few number of patients have been analyzed at the moment. It is of importance to note that the protocol to analyze archival material can resolve this matter in extensive retrospective studies, in which complete follow-up data can be included.

The mechanism that leads to increased HMG-I(Y) expression is unclear. Even more the mechanism by which HMG-I(Y) may interact of the five cases studied had a HMG-I(Y) expression just above the detection limit and the mean expression value for this group was 186 ± 157.

**Gleason Grade 3 Tumors.** In the four samples studied, three showed clear HMG-I(Y) expression, whereas one case was just above the detection limit. The HMG-I(Y) expression is measured after image analysis (954 ± 419). The signal was specifically located in the tumor cell areas.

**Gleason Grade 4–5 Tumors.** A high specific signal in tumor areas was found for this group. Seven samples were investigated and showed strong HMG-I(Y) expression on tumor tissue. The HMG-I(Y) expression obtained in this category was slightly higher than the one calculated for frozen sections (A = 2453 ± 1143; compare Table 1 and Table 3).

**Expression of HMG-I(Y) in Lymph Node Metastasis.** Ten lymph node metastases with preserved RNA were investigated. All specimen were paraffin embedded and fell within the Gleason grade (4–5) group. HMG(I) was highly expressed (Score A = 2000–2300 grains) in all cases except one, i.e., A = 260 grains.

Comparison of RISH results on frozen and paraffin-embedded material revealed (in most cases) slightly lower A values in paraffin material. However, this difference is not statistically significant. Thus, combining the RISH data from frozen and paraffin sections, it became clear that there is no major difference between frozen and paraffin material for RISH analysis.

**DISCUSSION**

In an attempt to identify molecular differences between aggressive and nonaggressive prostate cancer using Dunning R-3327 rat prostate cancer sublines as model, we found HMG-I(Y) to be overexpressed in highly metastatic sublines.

**Table 2. Statistical analysis of combined RISH data of frozen and paraffin sections**

<table>
<thead>
<tr>
<th>Differentiation</th>
<th>No. of cases</th>
<th>Mean of Score A ± SD</th>
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<tbody>
<tr>
<td>benign</td>
<td>4</td>
<td>ND</td>
</tr>
<tr>
<td>Gg (1–2)</td>
<td>5</td>
<td>187.0 ± 157</td>
</tr>
<tr>
<td>Gg (3)</td>
<td>4</td>
<td>955.0 ± 419</td>
</tr>
<tr>
<td>Gg (4–5)</td>
<td>7</td>
<td>2454 ± 1143</td>
</tr>
<tr>
<td>Meta</td>
<td>10</td>
<td>1900 ± 630</td>
</tr>
</tbody>
</table>

**Table 3. HMG-I(Y) expression in prostate cancer determined by RISH on paraffin-embedded material**

<table>
<thead>
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**Table 4. Relationship between HMG-I(Y) expression levels and stages**

| Correlation between stage and HMG-I(Y) expression over and below a threshold of 1300 (A score). |
|-----------------------------------------------|-----------------------------------------------|
| A < 1300                                      | T1    | T2    | T3    | T4    |
| A > 1300                                      | 3     | 7     | 6     | 5     |
with other factors in carcinogenesis is not yet understood. However, all these divergent functions attributed to HMG-I(Y) thus far can be explained by the assumption that the protein alters the structure of naked DNA or chromatin, allowing and/or facilitating the binding of other proteins. It has been recently shown that transcription factor NF-kB cannot activate transcription from its binding site PRDII unless an additional factor, which was identified as HMG-I(Y), was present (35). This synergistic effect is due to the interaction of HMG-I(Y) with A:T-rich sequence of PRDII through contacts in the minor groove, while NF-kB interacts with G:C-rich sequences at the two ends of PRDII through contacts in the major groove. Moreover, the fact that p50 and p65 subunits of NF-kB are members of the rel gene family may allow speculations about the indirect implication of HMG-I(Y) in cancer via oncogenes.

It has been also reported that HMG-I(Y) transcripts are highly expressed in fast proliferating undifferentiated cells (24), and it is related to a highly malignant phenotype rather than to neoplastic transformation (18, 36). Moreover, in the Dunning model system, HMG-I(Y) expression was specifically correlated with metastatic capacity and not with tumor-doubling time (11, 20). Similar findings were reported by Ram et al. (25) in mammary carcinogenesis. This study supports the previous data and, as in the Dunning R3327 rat prostate cancer model (11) and human cancer cell lines (1, 5, 6, 7, 14), HMG-I(Y) mRNA is increased in high grade malignant prostatic lesions. This is the first report on increased HMG-I(Y) expression in human cancers, i.e., in prostate cancer. The limited retrospective study performed in this report, however, does not allow definite conclusions concerning the clinical usefulness of HMG-I(Y) as a prognostic marker. A large scale study on archival material, together with a correlation analysis of HMG-I(Y) with survival, will be necessary before an accurate assessment of the role of HMG-I(Y) as a potential marker in prostate cancer can be made.

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REFERENCES

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