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Effect of androgen deprivation therapy on the expression of prostate cancer biomarkers MSMB and MSMB-binding protein CRISP3

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Running title:

ADT affects MSMB and CRISP3 expression

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ABSTRACT

Background: We have investigated the effects of short-term neoadjuvant, and long-term androgen deprivation therapy (ADT) on β -microseminoprotein (MSMB) and cysteine-rich secretory protein-3 (CRISP3) expression in prostate cancer patients. We also studied if MSMB expression was related to genotype and epigenetic silencing.

Methods: We investigated the expression of *MSMB*, *CRISP3*, androgen receptor (*AR*), *KLK3* and Enhancer of Zeste Homologue-2 (*EZH2*) in tissue from prostate cancer patients receiving (n=17) or not receiving (n=23) ADT before radical prostatectomy using an Affymetrix cDNA microarray analysis. *MSMB*, *CRISP3* and *AR* were studied in tissue from the same patients undergoing TURP before and during ADT (n=16). *MSMB* genotyping of these patients was performed by TaqMan PCR.

Results: *MSMB* and *KLK3* expression levels decreased upon ADT. Expression of *AR* and *CRISP3* were not affected by short-term ADT but were high in CRPC and metastases. Levels of *EZH2* were also high in metastases, where *MSMB* was low. Genotyping of the *MSMB* rs10993994 polymorphism showed that the TT genotype conveys poor MSMB expression.

Conclusions: *MSMB* expression is influenced by androgens, but also by genotype and epigenetic silencing. *AR* and *CRISP3* expression are not influenced by short-term ADT, and high levels were found in CRPC and metastases.

Keywords (3-6): microseminoprotein, PSP94, neoadjuvant, castration, tissue biomarker,

INTRODUCTION

Prostate cancer is the most common form of cancer in Western world males, and is also a leading cause of cancer related-deaths in males ¹. Patient prognosis will vary from a rapidly progressing disease with a high probability of death in a minority of patients, to a relatively indolent prostate cancer that can be controlled for the remainder of the patients life with little intervention in a majority of patients. Despite identification of a number of promising new biomarkers, there is still no absolute way of determining disease prognosis at the time of diagnosis. Inevitably, this leads to overtreatment of a large number of men for the benefit of the few who need it.

Attention has been focused on the predictive abilities of β -microseminoprotein (MSMB); also known as prostate specific protein of 94 amino acids (PSP94). This protein is second only to PSA as the most predominant protein in seminal plasma ^{2,3}. The function of MSMB is largely unknown, but *in vitro* and *in vivo* studies suggest it may be involved in apoptosis, cell mobility, vascularization, and tumor suppressor functions, all recently reviewed by Whitaker ⁴. The suitability of MSMB as a biomarker for prostate cancer has been raised by several groups during the last two decades. It has been reported that *MSMB* mRNA and protein expression is reduced in malignant prostatic epithelia compared to benign epithelia ⁵. In prostate cancer, the *MSMB* gene may be subjected to transcriptional silencing by methylation, mediated by Enhancer of Zeste Homologue-2 (EZH2), a Polycomb group member which is often overexpressed in CRPC ^{6,7}.

MSMB expression has been reported by several groups to be negatively associated with disease-free survival ⁸ and outcome ⁹. MSMB expression has also been proposed to be a significant prognostic indicator for prostate cancer progression under endocrine therapy ¹⁰.

Recently, the *MSMB* gene has gained further attention as one of the primary candidate prostate cancer susceptibility genes^{11,12}, and several causal risk alleles have been identified in the region upstream of the coding sequence¹³⁻¹⁵. Serum levels of MSMB, its binding protein PSP94-binding protein (PSPBP) and the ratio free/bound MSMB, have all been suggested as independent prognostic factors in prostate cancer¹⁶⁻¹⁹.

We have previously reported that the cysteine-rich secretory family (CRISP) family member CRISP3 also forms a high-affinity complex with MSMB in human seminal plasma²⁰.

CRISP3 has been found to be one of the most upregulated genes in prostate cancer compared to benign tissue^{21,22}, and was proposed to be a useful biomarker for prostate cancer^{23,24}.

Recently we reported that in a tissue microarray (TMA) with samples from 945 prostate cancer patients undergoing radical prostatectomy (RP), high CRISP3 and low MSMB expression were associated with poor outcome⁹.

As the impact of androgen availability on the expression of certain tumor biomarkers is currently unclear, we wanted to investigate how the candidate markers MSMB and CRISP3 are regulated by androgens during ADT and in progressive prostate cancer. First, we investigated the effect of short term neoadjuvant ADT on *CRISP3* and *MSMB* gene expression in primary prostate cancer, as well as in a limited set of prostate cancer metastases. CRISP3 and MSMB protein levels were then evaluated in serial tissue samples from a small but unique cohort of men with prostate cancer undergoing transurethral resection of the prostate (TURP) before and during long-term ADT. Finally, as MSMB expression may be differentially regulated in primary prostate cancers and in progressive disease, we also studied whether MSMB expression levels were related to a SNP in the *MSMB* promoter region and associated with epigenetic silencing of *MSMB* by EZH2.

MATERIALS AND METHODS

Sample preparation and data analysis of Affymetrix U95 human gene array

Microarray tissue samples were obtained as previously described²⁵. In brief, tissue samples were collected from patients undergoing therapeutic or diagnostic procedures at Memorial Sloan-Kettering Cancer Center, New York, NY. All samples were snap-frozen, histologically analyzed, and tissues containing approximately 60-80 % prostate cancer were manually dissected from the frozen block. Samples included 23 primary prostate cancer tissues from patients not receiving adjuvant therapy prior to RP, 17 primary prostate cancer tissues from patients collected at RP after three months of neoadjuvant ADT (monthly injections of 3.6 mg of goserelin and 250 mg flutamide three times daily). A limited set of 9 prostate cancer metastases (the secondary sites were in bone (two samples); lung (one sample) and lymph node (three samples)) and metastatic castration resistant prostate cancer (CRPC) (three samples, sites not specified) were also included. Details on patient characteristics have previously been described²⁵.

Gene expression analysis were performed as described previously²⁵. In brief, total RNA was extracted from frozen tissue by Trizol (Invitrogen, Carlsbad, CA), purified and evaluated for integrity. Complementary DNA (cDNA) was synthesized from total RNA, and gene expression analysis was performed using Affymetrix U95 human gene arrays with 63 175 probes for genes and expressed sequence tags (ESTs) as described by the manufacturer (Affymetrix, Inc., Santa Clara, CA). Samples were analyzed using Affymetrix Microarray Suite v4.0 as previously published²⁵.

Tissue specimens and clinical data from patients undergoing TURP

Formalin-fixed paraffin-embedded human tissue samples were obtained from 16 prostate cancer patients (mean age 70 years, range 52-78) undergoing repeated therapeutic TURP at the University Hospital Malmö, Sweden. Immunohistochemistry was performed as previously described⁹, using antibodies and dilutions as described in Table I. Descriptive characteristics of the patients are given in Table II. Samples were collected between 1971 and 2001. From each patient, samples were collected at diagnosis, prior to ADT and from a later TURP due to local tumor progression. Nine patients were subjected to medical castration with GnRH analogue, one patient was treated with flutamide monotherapy and five patients underwent bilateral orchiectomy. Mean follow-up time was 73 months (range 12-167 months) and mean time of hormonal treatment was 37 months (range 3-118 months). Patients were considered to have progressive disease and CRPC at the second TURP date as levels of serum PSA or acid phosphatase (PAP) were rising despite ongoing ADT. The study was approved by the local Ethic's Committee at Lund University, Sweden.

Genotype analysis

Paraffin embedded tissue from 13 out of the 16 patients undergoing TURP was available for genotype analysis. Fresh sections were microscopically studied, and tissue was manually punched out of the paraffin block with a 2 mm biopsy needle. DNA was isolated using the QIAamp DNA FFPE tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The rs10993994 polymorphism was determined using TaqMan primers and probes (Applied Biosystems, Foster City, CA), using 1 ng/μl template DNA in a reaction volume of 25 μl. The analysis was performed on a 7900HT fast real-time PCR system (Applied Biosystems) according to the manufacturer's instructions.

RESULTS

***MSMB* but not *CRISP3* transcript levels decrease in response to short-term neoadjuvant ADT**

Changes in expression of *MSMB* and *CRISP3* genes due to androgen deprivation were analysed using data from Affymetrix U95 gene expression microarrays. The clinical and pathological features related to the patients and tissue samples included in the study (patient age, preoperative serum PSA levels, Gleason score, pathological TNM stage, and recurrence) have been described earlier²⁵. *MSMB* expression was found to be high in primary prostate cancer from patients not receiving adjuvant ADT, and expression was significantly decreased upon ADT (Fig. 1; Mann-Whitney, $p < 0.001$). No significant reduction in gene expression levels was seen in either *CRISP3* or *AR* transcript levels in patients receiving neoadjuvant ADT, however both *CRISP3* and *AR* were highly expressed in metastatic prostate cancer lesions (Fig. 1). There was considerable inter-patient variability in expression of both *MSMB* and *CRISP3*. Interestingly, the *MSMB* expression level and decrease upon ADT was equal that of *KLK3*, a well-known target of androgen signaling (Fig. 1). As expected, *KLK3* gene expression levels decreased in the group receiving neoadjuvant ADT compared to the non-treated group (Mann-Whitney, $p < 0.001$). However, as in the case of *MSMB*, considerable inter-patient variability was detected.

Prostate cancer metastases express low transcript levels of *MSMB*, and high levels of *CRISP3*

A vast majority of prostate cancer patients develop CRPC within a few years following ADT. Such tumors often exhibit highly upregulated levels of AR though the signaling may be dysfunctional^{26,27}. In order to investigate *MSMB* and *CRISP3* transcriptional levels in

metastases, we studied gene expression in nine remote tumors, out of which three were from patients with CRPC.

Although caution is a prerequisite when interpreting small sample numbers, we found that *MSMB* expression is substantially decreased in metastatic tumors compared to primary prostate cancer, whereas *CRISP3* is expressed at high levels in metastases (Fig. 1).

Interestingly, *CRISP3* expression pattern appears to be somewhat similar to that of *AR* in that both transcripts are expressed at the same level and neither *AR* nor *CRISP3* is affected by ADT (Fig. 1).

Protein expression of MSMB and CRISP3 during ADT and prostate cancer progression

To evaluate the link between long-term ADT, progressing disease, and the expression levels of MSMB and CRISP3 proteins, we used a small but unique set of serially collected tissue samples from 16 patients undergoing TURP before and during ADT. At the time of the second TURP, all patients were in biochemical progress and considered to be in a CRPC stage (Table II). Tissue sections were immunohistochemically stained for MSMB, CRISP3, and AR, and protein expression was evaluated by a pathologist for both intensity and percentage of positive tumor cells. Representative staining is depicted in Fig. 2.

A majority of the patients had very low MSMB levels already at the time of the first TURP, and decreased expression was therefore not readily detected (Fig. 3). In the few patients with more than 50 % tumor cells positive for MSMB at the time of the first TURP, there was a dramatic decrease in expression at the time for the second TURP. No patient had more than 45 % tumor cells positive for MSMB at a CRPC stage. Tumor tissue from these CRPC

patients during long-term ADT showed higher levels of CRISP3 and AR compared with tissue from primary tumors from the same patient at the time of diagnosis (Fig. 3).

Increased expression levels of CRISP3 and AR were found in terms of an increased fraction of positive cells. MSMB and CRISP3 staining intensity did not change with progressing disease, but AR staining was stronger at the time for the second TURP. The decreased MSMB protein expression upon long-term ADT is corroborating the studies at the transcriptional level, and is also emphasizing that low MSMB expression is a feature of aggressive disease, whereas CRISP3 and AR show the opposite with high expression levels in CRPC.

Low MSMB protein levels are associated with the TT genotype of SNP rs10993994

The *MSMB* promoter contains a SNP reported to significantly affect *MSMB* gene expression. To investigate the impact of this SNP on *MSMB* expression in our experimental setup, we performed TaqMan genotyping of the rs10993994 locus on 13 out of the 16 patients previously immunohistochemically examined for MSMB expression. TaqMan genotyping identified the TT genotype previously associated with lower MSMB expression in two patients. Both patients were showing low levels of MSMB. The remaining 11 patients all had CC or CT genotypes, and taken as a group, a higher level of MSMB expression (Fig.3 and 4).

High transcriptional levels of the *EZH2* gene are associated with low MSMB levels in progressive disease and metastases

MSMB expression before and during ADT varies considerably between different patients, and can not be explained by either androgen availability or genotype. Previous studies have shown that methylation, mediated by the Polycomb group member EZH2, is yet another way by which *MSMB* expression may be regulated. Therefore, we analyzed the transcriptional levels

of *EZH2* in the Affymetrix U95 gene expression microarray described above. Although this is a limited sample set, we found that the *EZH2* gene was highly expressed in metastatic lesions from prostate cancer, where *MSMB* expression levels were very low. *EZH2* gene expression does not appear to be affected by neoadjuvant ADT (Fig. 1).

DISCUSSION

In the current study, we wanted to examine impact of short- and long-term ADT on prostate cancer outcome predictors *MSMB* and the *MSMB*-binding protein *CRISP3*. We show that short-term ADT significantly reduces both transcript and protein levels of *MSMB*, whereas *CRISP3* levels do not change. On the other hand, *CRISP3* is upregulated in parallel with *AR* and *KLK3* in progressive disease and during long-term ADT.

In concordance with studies conducted by others, our results support that *MSMB* expression decrease in patients receiving neoadjuvant ADT indicating androgen dependent expression²⁸. Importantly, and in contrast to *KLK3*, *MSMB* expression is continuously low in progressive and metastatic disease (Fig. 1). Rising levels of PSA is considered a hallmark for disease progression. This indicates that despite similarities in androgen effect on *KLK3* and *MSMB* expression in the normal prostate and primary prostate cancer, it is obvious that they are differentially regulated in progressive disease. This could be due to silencing of *MSMB* expression by *EZH2* mediated methylation of the *MSMB* promotor⁶. We find high levels of *EZH2* in metastatic lesions which is consistent of our findings of low *MSMB* expression in those tumors (Fig. 1). ADT did not appear to affect *EZH2* levels, thus methylation is unlikely linked to the rapid *MSMB* downregulation seen in hormonally treated primary prostate cancer.

Yet another way by which *MSMB* can be regulated is by a SNP in the promotor region. In our study, the TT allele was only seen in 2 out of 13 patients (Fig. 3 and 4). Both patients had very low levels of *MSMB*, but not lower than many patients carrying the CC or CT alleles. Despite much recent interest in the rs10993994 SNP genotype, our findings indicate that it does not fully account for the low *MSMB* levels generally seen in prostate cancer patients with progressive disease.

Our data showed that neoadjuvant therapy does not appear to affect expression of *CRISP3*. On the other hand, the high *CRISP3* expression in metastatic and recurrent tumors may be indicative of a role for *CRISP3* in the progression of prostate cancer.

In the present study, we find that *MSMB* expression may be regulated by several mechanisms, including androgen deprivation. In all, *MSMB* being suggested to act as a candidate tumor suppressor, it is most thought-provoking to note that according to this study, *MSMB* is downregulated by hormonal treatment and subsequently it may be silenced by different mechanisms. Our results favor the view of *MSMB* being a suitable marker for prostate cancer disease progression in CRPC.

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CONFLICT OF INTERESTS

The authors declare no conflict of interests.

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FIGURE LEGENDS

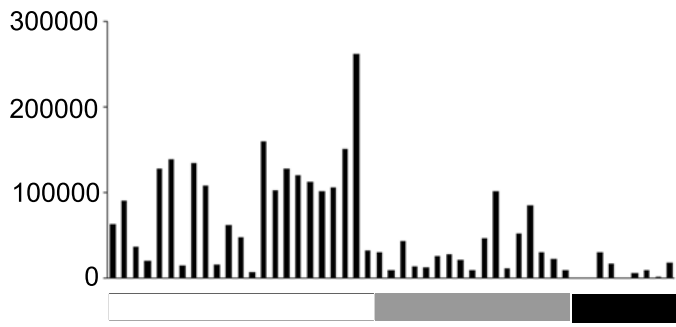
Fig. 1. Transcription profile changes before and after ADT in primary prostate cancer tumors and in metastases. Patients with primary prostate cancer undergoing routine surgery either received (n=17) or did not receive (n=23) neoadjuvant androgen deprivation therapy (ADT) prior to surgery. Transcription is represented as graphs and box plots for *MSMB*, *CRISP3*, *AR* (reproduced from *Am J Pathol* 2004, 164:217-227 with permission from the American Society for Investigative Pathology), *KLK3*, and *EZH2*. The cDNA array included two probe sets for *AR*, and the corresponding boxplot is based on the average.

Fig. 2. Immunohistochemical staining showing expression pattern of MSMB, CRISP3 and AR in TURP specimens derived from the same patient undergoing diagnostic TURP prior to ADT (upper panel), and later during ADT when TURP was performed due to local tumor progression at a CRPC stage (lower panel). Images were generated at an original magnification of 20x.

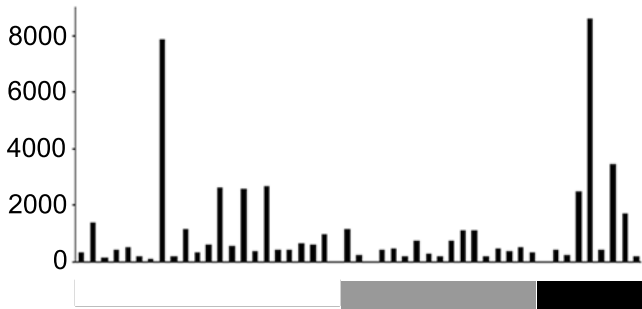
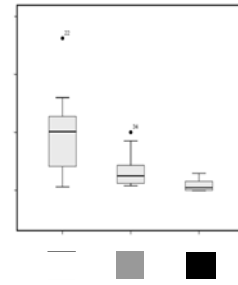
Fig. 3. Bar charts representing the fraction of cancer cells positively immunostained for MSMB, CRISP3, and AR in TURP samples from patients undergoing TURP before receiving any treatment (grey bars), and later at tumor progression during ADT (black bars). The rs10993994 SNP genotype for each patient is inserted below the upper chart, illustrating MSMB protein expression. *Tumor material missing.

Fig. 4. Correlation of the rs10993994 genotype and the corresponding percentage of MSMB positive tumor cells in tissue collected from patients undergoing TURP before initiation of ADT.

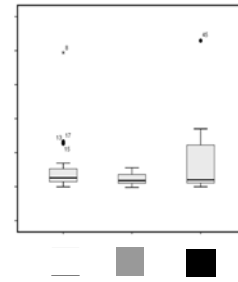
Figure 1



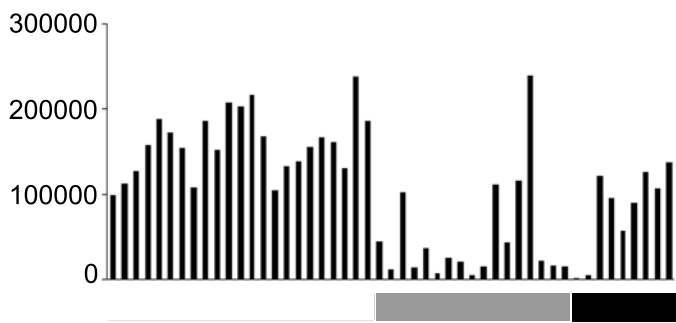
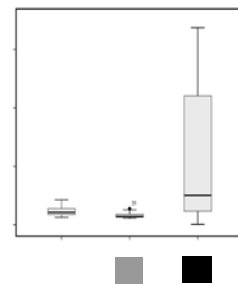
MSMB



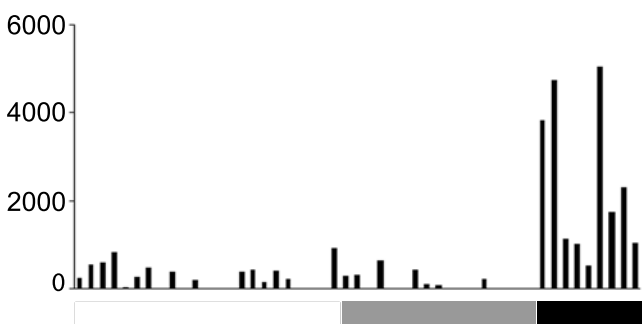
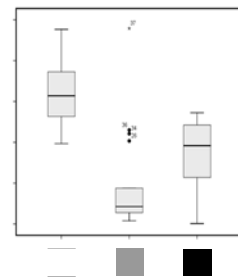
CRISP3



AR



KLK3



EZH2

Untreated primary prostate cancer
 Neoadjuvant ADT treated primary prostate cancer
 Prostate cancer metastases

Figure 2

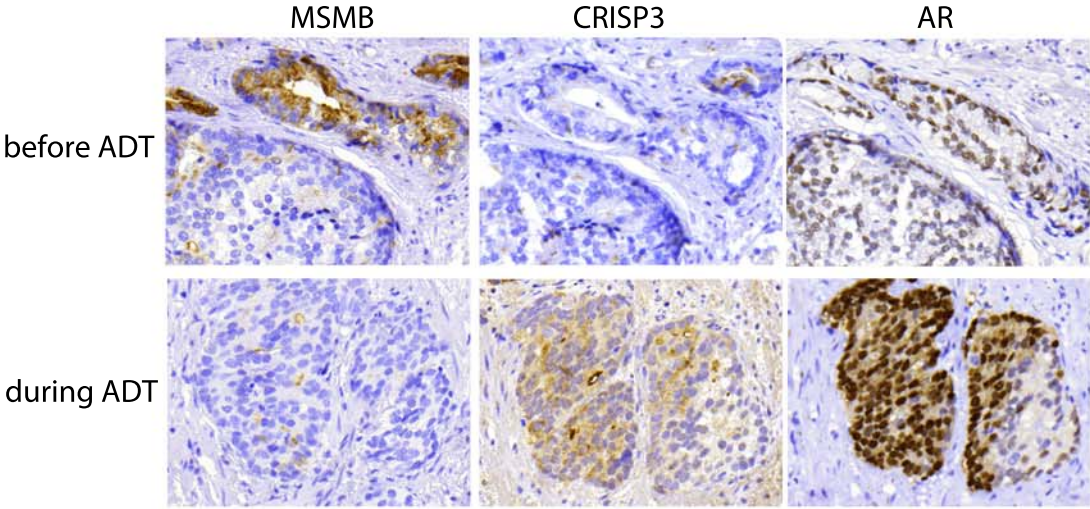


Figure 3

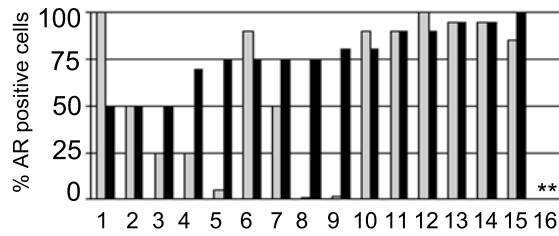
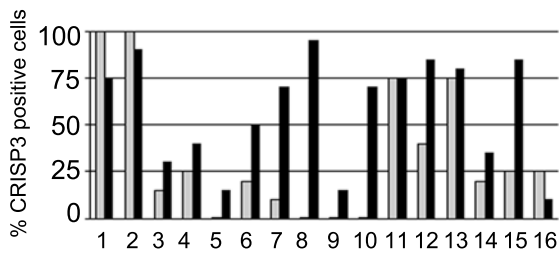
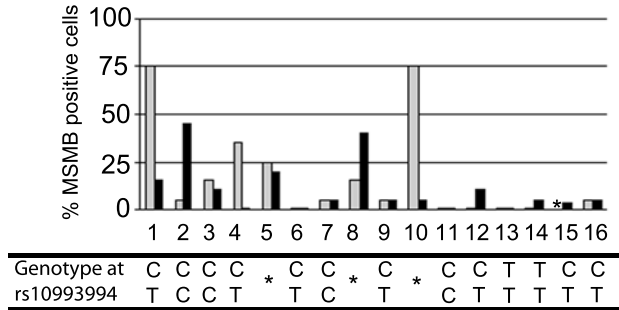


Figure 4

