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future outcome. Finally prostate cancer as a sec	, we have begun to develop a cond prognostic marker. W	a collagen-like prot e believe that a pa	ein that is u anel of such	pregulated in metastatic agents will eventually
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Introduction:

Although the development of the PSA test has improved our ability to diagnose prostate cancer, this has not been accompanied by improvements in prostate cancer prognosis. The goal of this project is identify molecules that may be useful as predictive markers for human prostate cancer and to develop these molecules for use in clinically relevant tests that can predict outcome and disease course in prostate cancer patients. Our approach has been to identify molecules that are upregulated late in the course of prostate cancer progression and to then test whether the expression of these markers correlates with disease outcome in patients. Our initial work has been focused on thymosin \$15, a molecule found in mid-high grade prostate cancers. We have been following two approaches to develop TB15 testing as a clinically useful approach. The first involves using immunohistochemistry to assay tissue sections from human prostates for the presence of TB15 and to then correlate the expression patterns with PSA failure, tumor recurrence, metastasis or mortality. The second approach has been to attempt to develop an ELISA test to detect TB15 in the urine or serum of prostate cancer patients in order to have a non-invasive assay. We have accomplished both of these goals and our early results do suggest that TB15 may have promise as a marker that can predict later tumor aggressiveness and metastatic potential. We believe that such a test could eventually be used to distinguish those patients at low risk of recurrence from those who are at higher risk. This information could be extremely useful in helping patients and their physicians to choose between the wide range of treatment options currently available.

Body:

In previous studies, by comparing gene expression among the Dunning R-3327 rat prostatic adenocarcinoma variants (1), we cloned a novel β -thymosin gene, thymosin β 15, which was expressed in highly metastatic variants, but not in poorly metastatic variant (2). The thymosin β family comprises very small, highly conserved and acidic proteins existing in many different animal species (3). The most abundant member of the family, thymosin β 4, which was originally isolated from calf thymus and postulated to play a role in thymic immune development, is present in all mammalian species, and along with the related family member thymosin β 10, is widely distributed in a variety of cell types. The main function of thymosins β 4 and β 10 is to bind monomeric actin and to retard actin polymerization (4)(5). Thymosin β 15 also binds monomeric actin and appears to regulate cell motility as transfection of antisense thymosin β 15 into rat prostatic carcinoma cells can significantly reduce stimulated cell migration (2).

Note: The figures for this progress report are contained in two presentations that are included in the Appendix and labeled Presentation 1 and Presentation 2.

When we investigated the pattern of expression of TB15 in human prostate cancer we found that low-grade prostate cancers (Gleason 3-5) generally did not express TB15 whereas high-grade tumors (Gleason 8-10) most often had high TB15 levels. What was most interesting, however, was that there was no discernable pattern of TB15 expression in mid-grade tumors. Some were positive for TB15 and some were negative (Presentation 1, Fig. 4). This raised the prospect that TB15 might represent a predictive marker in human prostate cancer, i.e., that tumors that expressed TB15 might be more

aggressive than tumors in which TB15 was not expressed. In Task 1 of this proposal, we further evaluated thymosin β 15's use as a potential biomarker that can function as an indicator of metastatic progression and disease outcome in human prostate carcinoma patients by a small-scale retrospective study.

Task 1. Continued Development of Thymosin β 15 as a Prognostic Marker in Human Prostate Cancer.

Goals:

- a. Continue to accumulate follow-up data on the original cohort of patients analyzed for TB15 expression in our initial studies.
- b. Collect and stain tissue specimens for a large (>200 patient) retrospective study correlating TB15 expression with tumor metastasis and patient outcome (Months 6-24).

Results:

A polyclonal antibody was raised against a peptide representing the 11 C-terminal amino acids of thymosin β 15. Synthesized peptide was coupled with a carrier, keyhole limpet hemocyanin (KLH), and injected into rabbits. Antiserum was affinity-purified over the C-terminal peptide coupled CNBr-activated sepharose 4B column. To test the specificity of the purified antibody, we performed Western analysis of the GST/thymosin β fusion proteins with the affinity-purified anti C-terminal antibody. The purified antibody reacted strongly with the GST-thymosin β 15 fusion protein, but did not cross react with GST-thymosin β 4, nor with GST alone. Additional antibodies were prepared to the Cterminal 11 amino acids of T β 15 by conjugating the peptide to keyhole limpet hemocyanin and injecting the conjugate into chickens. These antibodies showed both high titer and high specificity and did not recognize any other β thymosin isoforms. We used the affinity purified polyclonal thymosin β 15 antibody for immunohistochemical study of 150 human prostate carcinoma cases. Positive immunostaining was observed in the cytoplasm of carcinoma cells in neoplastic prostates but not in normal prostates and not in the stromal cells. Among the specimens investigated, poorly differentiated adenocarcinomas with Gleason scores 8-10 displayed the most extensive and intense thymosin β 15 immunoreaction, followed by moderately differentiated prostate carcinomas with Gleason scores of 6-7 in which some but not all carcinomas were TB15 positive. In some cases, specimens of PIN showed thymosin β 15 immunostaining, but usually to a lesser extent than the malignant lesions. In poorly differentiated and invasive prostate carcinoma, single cells invading the tissue stroma displayed intense staining. Well-differentiated carcinomas with Gleason scores generally showed no thymosin β 15 staining or very low levels of staining.

Thymosin β 15 staining levels for all prostatic carcinoma specimens are summarized in Presentation 1, Fig. 4. Specimens were scored as negative if less than 10% of the tumor tissue in each section showed staining of tumor cells, scored as positive (+) if staining was between 10% and 50%, and strong positive (++) if greater than 50% of the tumor tissue was stained. The results show a general correlation between thymosin β 15 staining and the Gleason scores. In most cases, high-grade tumors (Gleason scores 8-10) have a higher percentage of positive staining than low-grade (2-5) tumors. Interestingly, moderately differentiated prostate carcinomas with Gleason scores (6-8) could be divided into three groups according to the levels of thymosin β 15 expression. 17 (32%) out of 54 cases showed no expression of thymosin β 15, about 50% of cells in 24 (44%) cases expressed thymosin β 15, showing partial positivity, while 13 (24%) cases showed high levels of thymosin β 15 expression (more than 75% cells were positive).

These results suggest that thymosin β 15, a potential marker of aggressive prostatic carcinoma, assorts independently in mid-grade prostatic carcinomas.

To further investigate whether TB15 expression correlated with invasion and/or metastasis in human prostate cancer, we conducted a study in collaboration with Dr. John Petros of Emory University Medical School. Tissue samples taken from radical prostatectomy were analyzed for TB15 staining and the staining level (negative -, positive +, strongly positive ++) was recorded and then compared with the invasive or metastatic state of the tumor at the time of prostatectomy. Of the patients with non-invasive disease, 8 were negative, 2 positive and 1 strongly positive for TB15 expression as determined by immunohistochemistry. Patients with invasive but non-metastatic tumors were more generally positive (Presentation 1, Fig. 5). Finally, <u>all</u> of the 15 patients with metastatic disease diagnosed at the time of surgery were either positive (7 patients) or strongly positive (8 patients).

The follow-up on 26 of patients is summarized in Presentation 1, Fig. 8. Most striking is the data for the nine patients who have died of metastatic prostate cancer (DOD) in the past 3-5 years. Of these, none were negative, one showed positive staining and 8 displayed strongly positive for thymosin β 15 at the time of diagnosis. Of the 15 patients still alive with no evidence of disease (NED) 3-5 years following diagnosis, 8 were negative for thymosin β 15, three were positive and four were strongly positive for thymosin β 15 staining. Thus, of the patients who have follow-up data, all of those patients who were negative for thymosin β 15 staining at the time of diagnosis are still

alive with no evidence of disease. Because the population is still only shortly removed from their initial diagnosis, it will be most interesting to monitor whether those patients with no current recurrence but with extensive thymosin β 15 staining are indeed at greater risk to develop recurrent disease than those with low thymosin β 15 staining.

One of the most interesting analyses of this data is shown in Presentation 1, Fig. 7, which examines tumor recurrence as a function of PSA failure in 72 patients. The reappearance of PSA after removal of the prostate is a clear signal for extraprostatic growth of the tumor. In this figure, it can be seen that PSA failure occurs in 78% of those patients with intermediate T β 15 staining and 83% of those patients with strong T β 15 staining. This relationship is shown as a function of time in Presentation 1, Fig. 6 which shows an inverse Kaplan-Meier curve of the percentage of T β 15 positive and negative patients who go on to PSA failure over time. It can be seen that by 11 years, all of the T β 15 positive patients have positive PSA levels. In contrast only 25% of the T β 15 negative patients experience PSA failure. These data again suggest that T β 15 expression at the time of diagnosis correlates closely with aggressive disease as categorized by eventual PSA failure.

We have distributed our antibodies to thymosin β 15 to other laboratories and one such group has published the first data on outcomes in prostate cancer patients following analysis of T β 15 levels. Chakravarty and colleagues (7) studied 32 patients with clinically localized, moderately differentiated (Gleason 6/10) prostate cancer treated by external beam radiotherapy. All patients had clincal stage M0 disease at initial presentation, which was documented by bone scan. Their corresponding biopsy

speciment were stained immunohistochemically for T β 15 which was then correlated with the clinical outcome in a blinded test. The median follow-up was 6 years for all of the patients (range = 1-19 years). The outcomes of the 2 patients were grouped into three categories: patients with no evidence of disease (n=11), patients with PSA failure without documented bone failure (n=11) and patients with PSA failure and documented bone failure (n=10). T β 15 staining intensity strongly correlated with clinical outcome. Of those patients whose specimens stained 3+, 62% developed bone failure compared with 13% of those patients whose specimens stained 1+ (p=.01). The 5-year freedom from PSA failure was only 25% for those patients with 3+ staining compared with 83% for those with 1+ staining (p=.02). The results of this study were taken to demonstrate that T β 15 staining intensity can identify high and low-risk patients with moderately differentiated, clinically localized prostate cancer.

Task 2. Development of an assay to detect thymosin β 15 in human fluids such as urine or serum.

Goals:

- a. Develop additional antibodies for TB15 (Month 1-6)
- b. Develop a sensitive ELISA or sandwich ELISA assay for TB15
- c. Determine whether TB15 can be detected in human fluids (Month 6-12)

d. If TB15 is detected in human fluids, attempt to correlate TB15 expression in patient serum or urine with tumor metastasis and patient outcome (Months 12-30).

It is clear that the utility of the TB15 test would be increased if a non-invasive assay could be developed that could detect TB15 levels in the serum or urine of patients with prostate cancer. The β thymosins are, however, intracellular actin-binding proteins and

they lack a signal peptide for export making it less likely that they would be secreted into human body fluids. Interestingly, though, significant levels of thymosin ?4 are found in the serum of normal individuals (6) suggesting that members of the ?-thymosin family may be released into the circulation. We have therefore attempted to develop an ELISA for thymosin ?15 and to use it to detect TB15 in the urine of prostate cancer patients.

Results:

A polyclonal antibody was raised against a peptide representing the 11 C-terminal amino acids of thymosin ?15. Synthesized peptide was coupled with a carrier, keyhole limpet hemocyanin (KLH), and injected into both rabbits and chickens. Antiserum was affinity-purified over the C-terminal peptide coupled CNBr-activated sepharose 4B column. To test the specificity of the purified antibody, we performed Western analysis of the GST/thymosin ? fusion proteins with the affinitypurified anti C-terminal antibody. The purified antibody strongly reacted with GST-thymosin ?15 fusion protein, but did not cross react with GST-thymosin ?4, nor with GST alone. Using the chicken anti thymosin ?15 antibodies, we have developed an ELISA to TB15 that can detect TB15 at concentrations of 10-20 ng/ml.

The ELISA protocol is as follows:

TB15 (20 ng/well in 200 ?l) was adsorbed to Costar high-binding 96 well plates for 2 h at 37C. The wells were then washed with buffer containing 3 mg/ml BSA and then replaced with 200 ?l of a solution containing a 1:2000 dilution of chicken anti-TB15 antibody along with samples containing urine from prostate cancer patients or from normal controls that had been pre-incubated overnight at 4C. The plates were incubated for 1 h at 37C, then washed 3X, incubated for an additional 1 h with rabbit anti-chicken IgG and finally developed with the Vectastain ABC reagent. A standard curve from a typical assay is shown

in Presentation 2, Fig. 1a. Increasing concentration of TB15 added to the antibody solution results in a progressive decrease in signal with concentrations from 20 ng/ml - 1250 ng/ml.

Our studies have now revealed that TB15 can be detected in the urine of some prostate cancer patients. Figure 1b shows some of the raw data for a group of 11 prostate cancer patients. We have now conducted assays on 120 prostate cancer patients. The only criterion for inclusion in the study was that the patient had been diagnosed as having prostate cancer and was under active care by a urologist. Time following diagnosis ranged from days to decades. Positive TB15 values are ascribed to any patients whose urine TB15 level equals or exceeds 40 ng/ml as determined by extrapolation to a standard curve run on the same day.

As shown in Figure 1c, normal controls are generally negative with 31 patients negative and only 2 positive. Patients previously diagnosed with prostate cancer but disease-free at the time of urine collection were variable in TB15 production with 39 patients positive and 27 patients negative. Since many of these patients were only recently diagnosed, some of these individuals may go on to develop recurrent disease. Of 20 patients who had failed treatment as judged by increasing PSA levels at the time of urine collection, 18 had positive levels of TB15 in their urine whereas only 2 were negative. Finally of three patients who were bone scan positive, 2 were TB15 positive and one was TB15 negative.

We are pleased and surprised that we can detect TB15 in the urine of prostate cancer patients and are currently performing similar studies on a larger sample of 300 patients whom we intend to follow for a five year period. Certainly the trend appears to be that patients with tumor failure have an increased level of detectable TB15 in their

urine. We are now trying to duplicate this work using patient plasma as well as urine samples. Presentation 2, Figure 2 shows that the T β 15 ELISA assay can detect T β 15 in human plasma. Figure 2a goes on to show that some prostate cancer patients also have positive levels of T β 15 in their plasma. Most importantly, we wish to follow this current cohort of patients over time to determine whether newly diagnosed patients who have the highest circulating TB15 levels will be more likely to develop recurrent disease at some later time.

Task 3.

Goals:

Develop new antibodies for the collagen-like protein and obtain antibodies for lipocalin-2 and other potential prognostic markers from investigators (Months 6-12).

Correlate expression of these additional prognostic markers in human prostate cancer tissue specimens with tumor metastasis and patient outcome (Months 12-24).

Develop a sensitive ELISA assay for detection of the collagen-like protein in patient serum and urine (Months 18-24).

If possible, correlate production of the collagen-like protein in patient serum and urine with tumor metastasis and patient outcome (Months 24-30).

Results:

An additional member of the thymosin β family is upregulated in prostate cancer.

TB16. The principal members of the beta-thymosin family are thymosins β 4, β 10 and

 β 15. The molecules differ markedly in their C terminal 6 amino acids but are highly conserved in the rest of the molecule. Thymosins β 4 and β 10 are more closely homologous to each other than to thymosin β 15. Recently, a fourth mammalian thymosin has been described in the EST database as a beta thymosin isoform found in a

human neuroblastoma. This molecule has been named thymosin NB or thymosin β 16 and has been shown to be expressed in human neuroblastoma cell lines. When compared When compared with the other β -thymosins, thymosin β 16 shows closer homology to thymosin β 15 than to either thymosin β 4 or β 10. We have raised antibodies to full-length thymosin β 16 and show that it cross reacts with thymosin β 15 but not with thymosin β 4 or β 10. In contrast, antibodies to the c-terminal 10 amino acids of thymosin β 16 recognize only that isoform and none of the others. We have used the c-terminal antibodies for immunohistochemical staining of human prostate cancer specimens and showed that the distribution of thymosin β 16 is similar but not identical to thymosin β 15. The principal difference is that thymosin β 16 is present more frequently and in lower stage and grade tumors than thymosin β 15. One of our principal goals for the future will be to determine the differences between these two closely related isoforms with regard to function as well as to their patterns of expression in different human tumors.

We have also made some progress with our studies on a novel collagen-like gene that is upregulated in certain metastatic prostate cancer cell lines. This molecule has substantial collagen-like repeats along with novel intervening sequences. We have made peptide antibodies to this molecule in chickens using methods similar to those described above for thymosin β 15. Using these antibodies in immunohistochemistry on human prostate cancer sections, we find that the protein encoded by this new collagen-like gene (CLG) is also upregulated in aggressive prostate cancers.

The CLG protein is detected in predominantly in higher-grade prostate tumors (Gleason 6 and higher) with the most extensive staining in tumors with Gleason scores of 8 or higher). Further work is being conducted to extend this study and to correlate these

findings to patient outcome. We will also attempt to develop an ELISA for CLG that can be used to detect the protein in patient fluids.

Structural characterization of the thymosin β 15 gene. We have now performed detailed sequence analysis of the thymosin β 15 gene. The gene appears to exist as a single copy in the rat genome and is comprised of 3 exons distributed over 3 kilobases. The transcription start site was defined by primer extension analysis 30 basepairs upstream from the translation start site. Sequence analysis of the 5'-flanking region upstream of the translation start site revealed domains associated with promoter activity including a GC box, and SP1 site and an egr site. Analysis of the transcriptional activity of the 5'-flanking region was carried out using a luciferase reporter in rat prostate cancer cells. Thymosin β 15 sequences fused to the reporter in the appropriate transcriptional orientation consistently produced a ~50-fold increase in luciferase activity when compared to a promoterless luciferase control construct. Thymosin β 15 sequences fused in the opposite orientation failed to induce any significant level of reporter activity. This work has now been published (8) and is included in the appendix.

Potential role of thymosin β 15 in angiogenesis. Although we primarily see thymosin β 15 in the prostate carcinoma cells themselves, we also see some staining in the endothelial cells of small capillaries within the tumors themselves. At first we thought that this was non-specific staining but reports from the Kleinman lab at the NIH has made us think that the staining may be real and relevant. That group has shown that another thymosin family member, thymosin β 4, is capable of stimulating endothelial cell migration *in vitro* and angiogenesis and wound healing *in vivo* (9,10). We have confirmed this function for thymosin β 15 and show it be capable of both stimulation of endothelial cell migration and of stimulating angiogenesis in the mouse cornea. The

angiogenic activity of thymosin β 15 appears to be equal or better than that of thymosin β 4. Because thymosin β 15 not a component of the normal vasculature, we speculate that thymosin β 15 is released from tumor cells and is bound and perhaps internalized by vascular endothelial cells. It is also possible that tumor-derived thymosin β 15 potentiates tumor angiogenesis in tumors that produce this molecule.

Key Research Accomplishments:

- ♦ Identified novel potential prognostic markers for human prostate cancer.
- ♦ Developed specific antibodies for potential prognostic markers.
- \diamond Showed that thymosin β 15 expression correlates with invasiveness and metastasis in human prostate cancer.
- Showed that thymosin β15 expression correlates with patient outcome in human prostate cancer.
- \Rightarrow Developed a competitive immunoassay for detection of thymosin β15 in patient urine.
- \Rightarrow Developed a competitive immunoassay for detection thymosin β15 in patient plasma.
- ♦ Demonstrated that thymosin β15 was present in the urine of patients with recurrent prostate cancer.
- Showed that a novel β-thymosin isoform (thymosin $\beta 16$) is also upregulated in human prostate cancer.
- ♦ Identified a novel collagen-like gene as a potential prognostic marker in human prostate cancer.

Reportable Outcomes:

- Banyard J, Zetter BR. The role of cell motility in prostate cancer. *Cancer Metast. Reviews* 1999; 17:449-458.
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Hutchinson LM, Short SM, Becker CM, Bakthavachalam S, Zetter BR. Dual functions of Tβ15 in tumor progression: intracellular motility and extracellular angiogenic function. Abstacts of the Keystone Conference on Cell Migration and Invasion. May 2001.

Zetter BR, Hutchinson LM, Bao L. Thymosin β peptides as diagnostic tools in cancer. Abstacts of the International Conference on Immunology and Immunotherapy. May 2001.

Patents:

5,821,033 Human thymosin β15 gene, protein and uses thereof. 11/3/1998
6,017,717 Human thymosin β15 gene, protein and uses thereof. 11/25/2000
5,858,681 Method for prognosis of prostate cancer. 1/12/1999
6,150,117 Method for diagnosis of cancer. 11/21/2000

Individuals receiving pay from this grant

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Lere Bao Ph.D.

Christian Becker M.D.

Conclusions:

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 \cdot Thymosin β 15 is upregulated in malignant human prostate carcinomas but not in benign tissues.

• Poorly differentiated tumor cells show higher levels of expression of TB15.

 \cdot Patients with T $\beta15$ positive tumors at the time of diagnosis will likely experience PSA failure.

 \cdot T β 15 expression correlates positively with metastatic potential and poor prognosis.

 \cdot Thymosin $\beta15$ (T $\beta15$) is detectable in the urine and the plasma of prostate cancer patients

• Preliminary data suggests that TB15 concentration in urine samples is correlated to PSA failure and/or clinical metastatic disease.

• Clinical follow-up data strongly indicate that TB15 could be used as a molecular marker to predict the outcome of prostate cancer patients.

Future Directions:

• Expand retrospective immunohistochemistry study to include 500-1,000 patients.

• Monitor >300 patients continuously over 5 years to determine whether urinary levels of thymosin β 15 correlate with PSA failure and metastatic disease.

 \cdot Investigate the potential of thymosin β 16 and the collagen-like gene (CLG) as potential predictive markers of prostate cancer

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Appendix

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Table I. Thymosin β 15 (T β 15) – A new marker for metastatic prostate cancer

Thymosin β 15 is a protein that correlates with metastatic progression in human prostate cancer. We have investigated whether thymosin β 15 expression at time of diagnosis can predict disease outcome. Currently there is no independent marker that can predict outcome in human prostate cancer. A 5 year longitudinal study has been completed and the preliminary data has been gathered as summarized below.

- Of 79 patients (Gleason 4-9) with 5 year follow-up after surgery, 59 (75%) had PSA failure an indicator of tumor recurrence
- Tβ15 immunohistochemical staining was performed on paraffin sections of tumors collected immediately after surgery. Staining was determined to be positive or negative.

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TB15 (test)	Failure +	Failure -	Totals	
TB15+	53	7	60	
TB15-	6*	13	19	
Totals	59	20	79	

PSA failure (an indicator of disease recurrence)

Sensitivity: the proportion of subjects with the disease who have a positive test. In this case, 53/59 (90%) men with recurrent disease had a positive TB15 test at the time of diagnosis.

Specificity: the proportion of subjects without the disease who test negative. In this case, 13/20 (65%) men without recurrent disease had a negative test (*see note below).

Predictive value of a positive test: the probability that a person with a positive test actually has disease recurrence. Here, of the 60 men who tested positive, 53 have recurrent disease (88%).

Predictive value of a negative test: the probability of a person with a negative result does not have disease. Here, 13 of the 19 men with a negative result do not have disease (68%). However, of the 6 patients who were PSA failure+ and T β 15- (false negative findings), 4 had positive margins and therefore are likely to have had <u>local</u> (non-metastatic) recurrence of the tumor. Because T β 15 is a predictor of metastatic potential, there is no expected correlation between local regrowth and T β 15 staining. **When these patients are removed from the study the specificity of the test improves to* 13/15 or 87%.

There is a dramatic need for a good predictive test for likelihood of non-local recurrence of prostate cancer after surgery or radiation. The initial results from this retrospective test of patients with 5 year follow-up after treatment for prostate cancer indicate that thymosin β15 expression at time of diagnosis correlates with later disease outcome. Patients who have negative thymosin β15 tests at time of diagnosis may be candidates for watchful waiting. Patients who are thymosin β15 positive at time of diagnosis are at higher risk for tumor recurrence and may be candidates for more aggressive therapy.



ROLE OF THYMOSIN B15 AS A PROGNOSTIC MARKER FOR HUMAN PROSTATE CANCER



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BACKGROUND:

 Prostate cancer is the most common malignancy in American nen.

It is a slow-growing tumor with relatively low incidence of mortality.

olf it becomes metastatic, however, prostate cancer is often a fatal disease It is important to distinguish those patients whose tumors will progress to the metastatic state from those with little likelihood of causing mobility.

-Thymosin β15 (TB15) is upregulated in metastatic prostate cancer cells and has a potential as a predictive marker.

We stained human prostate cancer samples with a TB15 antibody and showed the correlate of TB15 expression with patients outcome.







Fig. 1 Expression of TB15 in various normal rat tissues. Northern blot was hybridized with TB15 cDNA probe.

In situ Hybridization



Fig. 2 In situ hybridization with antisense riboprobe for TB15 on human prostate cancer samples. A. TB15 mRNM is expressed in select prostatic interbultional neoplastic lesions (small arrow), but or others (ange arrow), B. invasive single tumor cells display interse TB15 expression.



Fig. 3 Immunostaining of human prostate carchoma tissues with an affinity-purified anti-TB15 Cummal peptide antibody. A Normalignant prostatic epithelia show on TB15 staining (large arrow), accessionally prostatic intraepithelia! incopiasia shows weak staining (smail arrow). B. Moderately differentiated prostate carchoma cash show helogeneous staining make prostate (smail arrow, positive; large arrow, negative). C. Poorly differentiated mesule prostate carchoma cells show shorog TB15 staining. D. Single cells intraeding stroma show intense cytopiasmic staining.

Summary of Immunostaining



4.4. The results are shown for 7 spectrams of benjan prostatic hyperplasia and 76 cancers with cleason scores of 4.10. Each symbol represents a spectram from a different protocular location, more than 70% of tumor cells showing positivity; padial, 10-75%, negative, isse than 10%.

Correlation with Invasion and Metastasis

Immunohistochemistry

 TB15 is upregulated in malignant human prostate carcinomas but not in benign tissues.

SUMMARY:

 Poorly differentiated tumor cells show higher levels of expression of TB15.

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Patients with TB15 positive tumors at the time of diagnosis will likely experience PSA failure.

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•TB15 expression correlates positively with metastatic potential and poor prognosis.

that TB15 could be used as a molecular marker to predict the outcome of prostate Clinical follow-up data strongly indicate cancer patients.



Fallure Curve for Margin Negative Patients

Fig. 5. Correlation of invasive and metastatic potential with initial immunostaining data for TB15 expression in human prostate cancers with 5-year follow-up.

TB15 Staining and Survival Status

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Fig. 6 Correlation of PSA failure data with initial immunostaining data for TB15 expression.

TB15 Staining and Cancer Recurrence

Fig. 8 Correlation of clinical 5-year follow-up survival data with initial immunostalning data for TB15 expression in human prostate carcinoma.

Dead from Metantania

Recurrent Disease

No New disease



REFERENCES:

Fig. 7 TB15 positive-prostate cancers show high percentage of recurrence.

TB15 staining Recurrence: 13%



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Detection of Thymosin Beta 15 in Urine and Plasma of Prostate Cancer Patients



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Background:

- Prostate cancer has highest incidence of solid tumors but relatively low mortality.
- •Thymosin Beta 15 (TB15) is upregulated in human metastatic prostate cancer.
- Immunohistochemically it has been shown to be a positive prognostic marker of metastasis.
 If detectable in human
- fluids it might also serve as a <u>diagnostic tool in</u> <u>human prostate cancer</u>.



Methods:

A polyclonal antibody (AB) was raised (chicken) against a peptide representing the 11 Cterminal amino acids of TB15. Urine and plasma samples from prostate cancer patients were randomly collected from two outpatient clinics.

An competition ELISA was developed by coating 96-well plates with GST-TB15 fusion protein. Samples from 344 patients and 20 normal controls were pre-incubated with an AB dilution and then incubated on the plates. Washing steps were followed by incubation with secondary AB (rabbit anti-chicken IgG). The reaction was then developed by an alkaline phosphatase reaction. Dilutions of synthetic TB15 pre-incubated with AB dilution served as a standard.

Results:





Conclusions:

- •Thymosin Beta 15 (TB15) is detectable in the urine and the plasma of prostate cancer patients.
- Preliminary data suggests that TB15 concentration in urine samples is correlated to PSA failure and/or clinical metastatic disease.
- •Thymosin Beta 15 could potentially serve as a <u>diagnostic tool in human</u> <u>prostate cancer.</u>

Citations: Bao L, Loda M, Janmey PA, Stewart R, Anand-Apte B, Zetter BR. Thymosin Beta 15: A novel regulator of turnor cell motility upregulated in metatstatic prostate

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Presentation 2

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The role of cell motility in prostate cancer

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Key words: prostate cancer, motility, invasion, metastasis

Abstract

Cell motility is a critical determinant of prostate cancer metastasis. The current review discusses the role for cell motility in metastatic dissemination, the evidence that prostate cancer metastasis is dependent on increased cell motility and describes the molecules whose expression has been shown to correlate with the increased motility that accompanies prostate cancer progression. These include receptors for growth factors and cytokines that regulate cell motility as well as intracellular proteins that interact with actin or that regulate signal transduction associated with cell motility. Motility related modulators include both positive regulators of cell movement that are upregulated during tumor progression and suppressors of cell movement that are down-regulated during progression. Because altered expression of such genes may determine the metastatic potential of any particular prostate tumor, we conclude that the appearance or disappearance of motility-related molecules could be used to aid in the diagnosis and prognosis of human prostate cancer.

Tumor cell motility is an important step in the progression of metastasis. Tumor cells migrate from the primary tumor, intravasate into tumor blood vessels, extravasate into the secondary tumor site, and migrate through tissue to establish a secondary metastatic site. Prostate carcinoma preferentially metastasizes to bone and once secondary sites are established, the tumor is aggressive and often associated with a poor prognosis. However, many prostate tumors will remain localized and confined to the prostate indefinitely. Currently there is no means of distinguishing these patients from those in which the prostate tumor will progress to metastasis. This is an important clinical problem as prostate cancer is the second leading cause of cancer death among men in the US. Current tests include prostate serum antigen (PSA), which is a useful determinant of tumor volume but does not reveal metastases or metastatic potential, and histologic grading by Gleason's score [1], which is most useful at the extremes of tumor differentiation. Prediction of the behavior of clinically localized and moderately differentiated prostate tumors remains difficult. As cell migration is a critical step in the metastatic cascade, knowledge of the molecules that enhance or suppress cell motility in prostate cancer cells may lead to new prognostic molecules, as well as new therapeutic targets for the prevention and treatment of metastatic prostate cancer.

A number of studies have demonstrated a strong correlation between tumor cell motility and metastasis. One of the earliest correlations between cellular motility and prostate cancer metastasis was made by Coffey and coworkers in the late 1980's. They utilized a series of prostate carcinoma cell lines of varying metastatic potential that had been established [2] from a spontaneously occurring prostate adenocarcinoma that had been carried by serial subcutaneous transfer in rats since 1963 [3]. This Dunning rat R-3327 prostate cancer cell model has been used extensively to demonstrate the relationship between metastasis and cell motility. Analysis of these cell lines using time lapse microscopy demonstrated that motility, quantified in terms of membrane ruffling, pseudopod extensions and cell translocation, correlates positively with metastatic potential [4,5], see Table 1. Fourier analysis, which determines the temporal and spatial changes in cell contours over time confirmed that cell motility correlates with metastatic potential in these cell lines [6]. The characteristic motility grades of the individual sublines are stably maintained both in vivo and in vitro [7].

Table 1. Relationship between motility and metastasis in the Dunning R3327 rat prostate adenocarcinoma cell lines [4–7]

Cell subline	Metastatic potential	Motility grade
G	low	low
Н	low	low
HIF	low	low
AT1	low	low
AT2	low	low
AT3	high	high
PAT2	high	high
AT6	high	high
MatLu	high	high
MATLyLu	high	high

Growth factor, cytokine and hormonal regulation of prostate carcinoma cell motility

The prostate tumor cell environment is complex, consisting of surrounding tumor cells, extracellular matrix and many other cell types, including endothelial cells, smooth muscle cells, stromal cells and specialized epithelial cells, such as basal and exocrine secretory cells and neuroendocrine cells. Each of these cell types may secrete factors that modulate prostate carcinoma cell behavior, such as proliferation, differentiation and motility. Upregulation of growth factor synthesis or secretion, or altered expression of growth factor receptors on prostate carcinoma cells has the potential to modulate cell motility. For example, production of epidermal growth factor (EGF) and transforming growth factor- α (TGF α) is increased in prostate carcinoma tissue and cell lines [8], and EGF is a potent chemoattractant for several prostate carcinoma cell lines [9]. EGF receptor levels are also increased on prostate carcinoma cells [8], and overexpression of wild type EGF receptor on DU-145 human prostate carcinoma cells increases cell invasion through Amgel (a human basement membrane matrix) [10]. In addition, expression of a truncated, mitogenically active, but motility deficient EGF receptor in DU-145 cells inhibits the metastatic ability of these cells in vivo [11], demonstrating that motility, not proliferation, is the key event influencing EGF receptor-induced metastasis. Thus prostate carcinoma cells may increase their motility when they are proximal to EGF-secreting cells. Other factors may also influence EGF or EGF receptor expression. IFN- γ downregulates EGF receptor expression and thereby decreases invasion of DU-145 prostate carcinoma cells

through Matrigel (a tumor basement membrane matrix) [12]. Together, these results suggest an important role for EGF receptors in maintaining the motile phenotype of metastatic prostate cancer cells.

Autocrine motility factor (AMF), a prostate tumor cell secreted factor [13], also affects motility of prostate carcinoma cells. Tumor cell migration in response to AMF has been associated with metastatic potential. PC-3M is a highly metastatic subline of the human prostate carcinoma cell line, PC-3, that was established from a human prostate cancer liver metastasis. PC-3M exhibits a higher response to AMF, in terms of phagokinetic track motility analysis and accelerated closure of a experimentally wounded cell monolayer, than the parental PC-3 cells [14]. The AMF receptor, gp78, is differentially expressed on the membranes of PC-3 human prostate carcinoma cells and their highly metastatic subline PC-3M, indicating that receptor expression may be responsible for the differential response of these cells to AMF [14].

Additional growth factors also modulate prostate carcinoma cell motility. These include the transforming growth factor- β (TGF β), which stimulates migration of MAT-LyLu cells, a highly metastatic subline from the Dunning Rat R33327 prostate carcinoma model, across a porous membrane in an uncoated transwell assay [15]. TGF β may be produced by metastatic prostate tumor cells, as shown for the highly metastatic AT3 rat prostate adenocarcinoma cell line [16]. However, TGF β receptors I and II are downregulated in both primary prostate carcinoma and metastatic lesions [17], suggesting that advanced prostate cancer may eventually show TGF β insensitivity.

Prostate carcinoma frequently metastasizes to bone, a tissue rich in the insulin-like growth factors (IGFs), IGF-I and IGF-II, which are known to stimulate tumor cell movement. Therefore the effect of IGFs on prostate carcinoma cell motility may be important in metastasis. In fact, the chemotactic migration of prostate carcinoma cells is stimulated by either IGF-I or IGF-II [18]. The effect of IGFs on prostate carcinoma cells may also be modulated at the receptor level, as shown for EGF and TGF β , as the insulinlike growth factor-binding proteins (IGFBPs) are differentially expressed in prostate tumors with high and low Gleason's scores, indicating changes with disease progression [19]. In contrast, two bone-associated growth factors, tumor necrosis factor- α (TNF α) and interleukin-1ß (IL-1ß), decrease human prostate carcinoma cell chemotaxis [18] suggesting that the bone

microenvironment contains both positive and negative modulators of prostate cancer cell motility.

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Other cell types present in the prostate tumor environment include neuroendocrine cells. These cells can secrete bombesin, a gastrin-releasing peptide, which increases migration of PC-3 human prostate carcinoma cells [20]. Neuroendocrine cell function includes the secretion of prostatic hormones, which may also influence tumor motility. For example, calcitonin, a hormone involved in calcium regulation increases the chemotactic migration of LNCaP human prostate carcinoma cells [21]. Parathyroid hormone (PTH)-related peptide (PTHrP) is produced by prostatic neuroendocrine cells, and is overexpressed in prostate cancer tissue and cell lines [22]. PTH is a calcium regulating hormone which may be present at sites of prostate cancer bone metastasis that increases chemotactic migration of the human prostate carcinoma cell lines, DU-145 and PC-3 [21]. These experiments suggest that prostate cancer cell motility, and possibly metastasis, could be inhibited by agents that block calcium transients. An additional hormone secreted by the prostate is the androgen, dihydrotestosterone (DHT) which increases translational motility of LNCaP cells [23]. The hormonal status of the prostate, which may change with tumor progression, may thus influence cell motility, suggesting that hormonal therapy for prostate cancer may modulate cell motility and metastasis, in addition to affecting proliferation.

Prostate stromal cells secrete a nerve-growth factor (NGF)-like protein which stimulates the chemotactic and chemokinetic migration of the human prostate tumor cell lines, TSU-Pr1, PC-3 and DU-145 [24]. NGF stimulates DU-145 human prostate carcinoma cell invasion into Matrigel [25]. Hepatocyte growth factor or scatter factor (HGF/SF) is also produced by fibroblasts, and increases cell scattering and Matrigel invasion by DU-145 human prostatic carcinoma cells [26]. These data suggest that the stromal cells surrounding the prostate tumor may influence their migratory behavior.

An additional mechanism of control of prostate tumor cell migration at the primary site may be the paracrine secretion of motility inhibiting factors by tumor cells. For example, the non-motile, nonmetastatic G subline in the Dunning R-3327 rat prostate adenocarcinoma system secretes proteins capable of inhibiting motility of the highly motile and metastatic MAT-LyLu subline [27]. It is clear that numerous cell types in the prostate carcinoma environment may secrete factors which can both stimulate and inhibit motility, directly or via modulation of tumor cell receptor expression. Control of these effects may be a mechanism through which tumor cell dissemination may be reduced or prevented, and thus may represent potential targets for therapeutic agents.

Matrix modulation of prostate cancer cell motility

In addition to growth factor and cytokine mediators, the surrounding matrix may affect prostate carcinoma cell motility. Cell adhesion is critical for tumor cells to migrate, and therefore changes in the extracellular matrix may be expected to modulate tumor cell motility. In fact, Matrigel, collagen I and laminin all increase translational motility of LNCaP cells [23], and fibronectin stimulates DU-145 prostate carcinoma cell migration in the Boyden chamber assay [28]. Interestingly, culture of a transformed rat prostate epithelial cell line (NbMC-2) in Matrigel results in altered morphology and increases chemokinetic migration and invasive potential [29]. These data indicate that the extracellular matrix may influence both motility and metastatic progression of prostatic carcinoma cells.

Cell-matrix interactions are mediated via integrin receptors, and changes in integrin receptor expression have been associated with prostate cancer progression. In prostate tumors, increased $\alpha 6$ integrin expression correlates positively with the progression of prostate carcinoma cells to a motile and invasive phenotype. DU-145 cells selected for high (DU-H) and low (DU-L) $\alpha 6$ integrin expression, demonstrate similar adhesion, but the DU-H cells (with 4-fold higher $\alpha 6$ integrin expression), are 3 times more motile on laminin and are more invasive in vivo [30]. Integrin receptor aIIb β3 may be important in tumor cell dissemination as blocking antibodies inhibit prostate carcinoma cell metastasis [31]. Alternative splicing of integrin receptors is an additional mechanism through which tumor cell interaction with the extracellular matrix can be modulated. Alternative splicing of α IIb β 3 can result in a truncated receptor which lacks the transmembrane region and cytoplasmic tail of the α IIb light chain. This isoform is detected in the prostate carcinoma cell lines, PC-3 and DU-145, but not in normal prostate epithelial cells [32]. Loss of expression of the α 4 integrin subunit also correlates with the capacity of prostate carcinoma cells to invade through Matrigel [33]. In conclusion, these data suggest that both the extracellular matrix, and receptors mediating cell-matrix contact may play important roles in the control of prostate carcinoma cell movement.

Proteases and cell motility

Secretion of proteases by prostate cancer cells, resulting in degradation of the extracellular matrix, may allow cell migration and invasion into tissues at either the primary site or secondary metastases. The secretion of plasminogen activators (PA), a family of serine proteases, is associated with metastatic potential of prostate carcinoma cell lines. A more aggressive, highly metastatic subline of PC-3 cells, PC-3CALN, secretes 3.5 times more PA activity than the parental PC-3 cells, and invades Matrigel to a greater extent than PC-3 cells. PC-3 metastases also have greater PA activities than the corresponding primary tumor [34], supporting a role for PA in metastasis. Expression of urokinase plasminogen activator (uPA) also correlates with metastatic potential in the Dunning R-3327 rat carcinoma model [35]. Additional extracellular matrix-degrading enzymes, including elastase, a chymotrypsin-like activity, and prostatic hyaluronidase are also elevated in highly metastatic prostate carcinoma cell lines [36,37]. Finally, reduced expression of matrix metalloproteinase-9 (MMP-9 or Gelatinase B) decreases prostate carcinoma cell metastasis while not affecting the growth of the primary tumor [38]. Thus, increased protease activity in prostate carcinoma cells may represent an important mechanism through which cells escape the primary tumor and facilitate increased motility and metastasis.

Clinical compounds affecting prostate carcinoma cell motility

A number of clinical compounds affect prostate carcinoma cell motility. For example, the chemotherapeutic agent Pentosan inhibits motility of the highly metastatic Dunning rat carcinoma subline, MAT-LyLu [39]. Pentosan affects cell-matrix interactions and increases cell ruffling while inhibiting lamellipodal extension and reducing translational migration. Estramustine, an estradiol and nor-nitrogen mustard conjugate antimicrotubule drug, which is used to treat hormonerefractory advanced prostate cancer, inhibits migration of DU-145 prostate carcinoma cells from a cell aggregate and inhibits cell invasion of chick heart fragments [40]. Finally, N-(4-hydroxyphenyl) retinamide (4-HPR or Fenretinide) inhibits migration of PC-3 and TSU-Pr1 human prostate carcinoma cells [41]. Inhibition of cell motility by these clinical compounds may represent an important mechanism through which they prevent metastasis *in vivo*.

Genetic markers of prostate carcinoma encode motility-related proteins

In the past decade, several novel technologies, such as subtractive hybridization, differential display and, most recently, gene arrays, have allowed the identification of several genes whose expression is altered in tumor progression. When these techniques have been applied to the study of prostate cancer, they have not only revealed new metastasis-enhancing and metastasis-suppressing genes, but they have also strengthened the correlation between metastasis and motility. In nearly every case, the molecules that promote prostate cancer metastasis also promote prostate cancer cell motility. Similarly the molecules found to suppress prostate cancer, suppress prostate cancer cell motility, as shown in Table 2. Thus, it appears that the process of prostate cancer progression involves the upregulation of motility promoting genes along with the loss or mutation of motility suppressing genes.

We used differential display to identify a novel gene, upregulated in highly metastatic Dunning Rat carcinoma cells. Thymosin β 15 (T β 15) is a 5.3 kDa member of the beta-thymosin actin-binding protein family. It is expressed in human prostate cancer tissue, with a pattern reflecting increased disease progression as assessed by Gleason grading but is not detected in normal prostate epithelium or in other prostate lesions, such as benign prostate hyperplasia (BPH). We have shown a direct correlation between prostate carcinoma cell motility and T β 15 expression. Highly motile and highly metastatic AT3.1 cells from the Dunning rat carcinoma model express high levels of TB15, and transfection of antisense T β 15 is sufficient to markedly decrease cell migration [42]. Thus, a molecule that was identified as being upregulated in metastatic prostate cancer cells is necessary for the increased motility of

Molecules		Effect on motility	Association with or effect on metastasis
Growth factors and cytokines	EGF	†	 ↑
	AMF	↑	↑
	tgfβ	↑	nd
	IGF-I and IGF-II	↑	nd
	NGF	↑	nd
	HGF/SF	↑	nd
	bombesin	↑	nd
	TNFα	\downarrow	nd
	IL-1β	\downarrow	nd
Hormones	calcitonin	↑	nd
	PTH	1	nd
	PTHrP	nd	↑
	DHT	↑	nd
Membrane receptors	α6	↑	↑
	α4	↑	nd
	αΠbβ3	↑	↑
	CD44	\downarrow	\downarrow
	E-cadherin	nd	\downarrow
Proteases	plasminogen activators	↑	↑
	elastase	nd	↑
	hyaluronidase	nd	↑
	MMP-9	nd	↑
Actin-binding proteins	τβ15	↑	↑
	PTEN	\downarrow	\downarrow
	Tmel	nd	\downarrow
Tumor suppressor genes	bcl-2	↑	↑
	KAI-1	\downarrow	\downarrow
	Rb	\downarrow	\downarrow
Intracellular signaling proteins	ras	↑	1
	GAPDH	↑	1
	PKC-zeta	\downarrow	\downarrow

Table 2. Summary of functional effects of factors on prostate cancer cell motility and metastasis

 $\uparrow =$ increase; $\downarrow =$ decrease; nd = not documented.

these cells. The finding that T β 15 expression correlates with aggressive prostate cancer in humans suggests that it may be useful as a prognostic marker in human prostate cancer. We are currently developing an immunoassay that can detect low levels of T β 15 in the serum or urine of prostate cancer patients.

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Bcl-2 may be an additional positive marker of prostate carcinoma progression. Expression of bcl-2 in prostate carcinoma correlates with increased recurrence of disease [43]. A role for bcl-2 in cancer cell motility was shown in a breast carcinoma cell line, where overexpression of bcl-2 increased both migration and metastasis [44].

In contrast, the tumor suppressor gene PTEN (Phosphatase and Tensin homolog deleted on chromosome 10) is deleted in prostate cancer cell lines [45]. PTEN is a phosphatase with similarity to the cytoskeletal protein, tensin, which binds to actin at focal adhesions and is phosphorylated upon integrin binding. PTEN overexpression inhibits fibroblast cell migration in a wound scrape assay; antisense PTEN expression stimulated cell migration [46]. The migration inhibiting activity is mediated by the interaction of PTEN with focal adhesion kinase (FAK), resulting in reduced focal adhesion formation and cell spreading in a phosphatase-dependent manner. FAK overexpression partially antagonizes the effects of PTEN. Indeed, FAK is highly expressed in metastatic prostate carcinoma [47], and studies have demonstrated a role for FAK in cell migration [48]. Consequently deletion or mutation of PTEN in tumors should result in increased tumor cell motility.

KAI-1 is an additional tumor suppressor gene identified in prostate carcinoma. KAI-1 is a 267 amino acid protein, expressed in normal prostate tissue, but reduced in the prostate carcinoma cell lines PC-3, LNCaP, TSU-Pr1, DU-145 and AT6.1 [49]. Overexpression of KAI-1 in AT6.1 cells suppresses lung metastasis following subcutaneous inoculation, with no effect on the growth rate of the primary tumor. This suggests that the loss of KAI-1 may play a role in metastatic prostate cell dissemination, rather than tumor growth. Like PTEN, KAI-1 is also a suppressor of cell motility (J. Isaacs, personal communication). Thus, two potent cancer metastasis suppressors both function to suppress cell motility. Loss of these genes during prostate cancer progression consequently results in a highly motile, metastatic phenotype.

Loss of the Retinoblastoma (Rb) tumor suppressor gene also correlates with prostate carcinoma progression [50]. Rb-defective cell lines exhibit increased migration across Matrigel in a Boyden chamber assay [51], demonstrating a negative role for Rb in cell motility, as seen for the metastasis suppressor genes, PTEN and KAI-1. Because of its role of a suppressor of both proliferation and migration, Rb may function as both a tumor growth suppressor and a metastasis suppressor.

CD44 may be an additional tumor suppressor gene inactivated in prostate cancer. CD44 is a transmembrane glycoprotein, involved in cell adhesion by acting as the receptor for the extracellular matrix molecule, hyaluronic acid. CD44 can be alternatively spliced and loss of either the standard or variant isoforms is associated with prostate cancer progression [52]. Downregulation of CD44 expression also correlates with metastatic potential in the Dunning rat prostate carcinoma model, and overexpression in the highly metastatic AT3.1 cells reduces lung metastasis without affecting proliferation rate [53]. Similarly, overexpression of CD44 in PC-3 human prostate carcinoma cells, which express very low CD44 levels, inhibits metastasis in vivo [54]. Interestingly, inhibition of metastasis by CD44 overexpression was recently shown to be independent of hyaluronan binding [53], suggesting that CD44 may mediate an additional function required in metastasis.

Lastly, decreased expression of E-cadherin, a member of the family of epithelial cell-cell calciumdependent adhesion molecules, is found in invasive sublines of the Dunning R-3327 rat prostate adenocarcinoma model [55]. Decreased cell-cell contact with surrounding prostate epithelial cells may facilitate carcinoma cell dissemination by allowing cells to disaggregate and separate from the primary tumor. ...

The relationship between cell motility and metastatic potential has been demonstrated for numerous other marker genes, including nm23 [56], TIMP-2 [57], and MTS-1, a member of the S100 family of Ca²⁺ binding proteins [58]. A number of tumor markers have been identified in prostate carcinoma, including MTS-1 (CDKN2) [59,60], DCC, APC/MCC, WAF1/CIP1 [61], and PTI-1 [62], but their role in prostate carcinoma cell motility has not yet been documented. The roles of genetic tumor markers in prostate cancer have been previously reviewed [63,64]. We expect that the majority of metastasis-related genes will encode proteins that have some influence on tumor cell motility.

Modulation of intracellular function in prostate cancer

Cell migration depends, in part, on the regulation of intracellular calcium fluxes. The concentration of extracellular calcium modulates PC-3 human prostatic carcinoma cell migration in the Boyden chamber assay, whereas culturing cells in low calcium decreased invasion across a basement membrane-coated filter. Exposure to higher calcium levels stimulates invasion in a dose-dependent manner [65]. Several actin-binding proteins that influence cell movement are sensitive to local calcium concentrations. For example, human epithelial tropomyosin (Tme1), a calcium dependent actin-binding protein, is downregulated in human prostate carcinoma cells compared to normal prostate epithelium [66]. Down-regulation of tropomyosin with increased tumorigenicity indicates that tropomyosin function may be required for regulation of the actin cytoskeleton during cell motility.

The control of ionic influx affects cell physiology, and altered expression of cell surface transporter proteins is associated with progression of tumor cells to a motile, metastatic state. Metastatic ability has been correlated with the expression of sodium channel proteins in prostate carcinoma cells. Cell transfection of rat prostatic carcinoma genomic DNA also results in elevated levels of sodium channel protein expression [67]. These authors suggest that either intracellular ionic homeostasis or membrane electrophysiological changes may explain the observed effect on cell invasion.

Intracellular signaling molecules mediate the transduction of growth factor and extracellular matrix

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signals, and these signals modulate prostate carcinoma cell motility and metastasis. It is now well established that deregulation of signaling molecule expression can affect cell motility. The expression of the small GTPase p21 *ras* shows a strong positive correlation with histologic grade in prostate cancer tissues [68], and expression of v-*ras* in the poorly motile, poorly metastatic AT2 Dunning rat prostate carcinoma cell line increases motility and metastasis [69]. Increased expression of Ha-*ras* is also associated with increased metastasis in one lineage of the Dunning R-3327 rat prostatic carcinoma model [70].

Expression of the signaling molecule protein kinase C (PKC)-zeta negatively correlates with prostate cancer cell motility. PKC-zeta is downregulated in highly metastatic Dunning rat carcinoma cell lines. Conversely, PKC-zeta overexpression in highly metastatic MAT-LyLu cells decreases invasion through Matrigel and the formation of lung metastases *in vivo* [71], suggesting that PKC-zeta may represent an important intracellular signaling checkpoint in the control of prostate carcinoma metastasis.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression is positively associated with increased motility and metastasis in the Dunning rat prostatic carcinoma model [72]. GAPDH has diverse biological functions and the mechanism by which increased expression affects cell motility has not yet been reported.

The experimental record thus demonstrates that several intracellular molecules that are capable of modulating tumor cell motility also influence metastasis. The loss and acquisition of these motility-related proteins is a critical determinant of metastatic potential.

Conclusion and therapeutic implications

Tumor cell motility is a critical step during the progression of tumor cells to the metastatic phenotype. As we have reviewed, the motility of prostate carcinoma cells can be influenced by numerous factors in the tumor environment, including growth factors, hormones and extracellular matrix. Blocking migration stimuli or delivering motility inhibitors are obvious steps for possible clinical intervention. For example, if means could be found to prevent the loss of motility/metastasis suppressing proteins such as PTEN or KAI-1, or to prevent the gain of motility/metastasis enhancing molecules such as thymosin β 15, progression to the metastatic state could be delayed.

Perhaps the most exciting area of current research that may lead to new clinical tools and therapeutic agents is the identification of genes that are differentially expressed with prostate cancer progression. Expression of molecules that either promote or suppress cell motility and metastasis define a specific axis through which tumor progression might be monitored. A large proportion of prostate tumors remain localized to the prostate indefinitely and for such patients 'watchful waiting' may be more appropriate than radical prostatectomy and/or radiation therapy. Assays detecting the presence or absence of genetic markers of disease progression, such as these motility-modulating molecules, would provide a means of characterizing the metastatic potential of the prostate tumor, which could in turn influence treatment decisions. The ability to distinguish indolent tumors from aggressive tumors could have the potential to individualize cancer treatment for each patient.

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THYMOSIN β15 AS A PROGNOSTIC MARKER FOR HUMAN PROSTATE CANCER

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Prostate cancer is the commonest cancer in men and the second leading cause of cancer death in American men. The widespread use of prostate specific antigen (PSA) test has greatly improved the earlier detection of human prostate cancers. However, this early detection does not help much to predict which tumors will progress to the metastatic diseases. To search for molecules that could be used as prognostic markers for prostate cancer, we compared gene expression among Dunning rat prostatic carcinoma cell lines with varying metastatic potential and cloned a gene called thymosin beta 15 (TB15), a new member of the thymosin beta family, from a metastatic subclone. TB15 was not detected in most normal adult rat and human tissues, including prostate. In situ hybridization and immunohistochemical staining of human prostate specimens showed that both TB15 mRNA and protein levels were elevated in invasive and metastatic tumors and correlated positively with the Gleason grade, a common histological grading system of prostate cancer. Up to 5 years follow-up data from the patients we could obtain showed that all patients who died of metastatic prostate cancer had positive staining for TB15 at the time of diagnosis. In contrast, patients who were negative for TB15 are still alive with no evidence of disease. These data suggest that TB15 could serve as a molecular marker that is able to distinguish prostate cancers destined to progress to lethal metastatic disease from those with little likelihood of causing morbidity.

Thymosin β peptides as diagnostic tools in cancer. Bruce R. Zetter, Lloyd Hutchinson and Lere Bao. Children's Hospital, Harvard Medical School, Boston, MA USA. The β thymosin family consists of at least 17 different members. In any species, several different β thymosins will be expressed, each independently regulated to appear in different cell types and at different times in development. Several β thymosins, including thymosin β 4, β 10, β 15 and β NB have been shown to be upregulated in certain human cancers. We have made specific C-terminal antibodies that distinguish between thymosins β 4, β 10, β 15, and β NB. Using prostate cancer as an example, we have shown that thymosins β 15 and β NB are elevated in aggressive human prostate cancer whereas thymosin β 4 levels are reduced. Additionally, we find that the upregulation of thymosin β 15 in primary prostate cancer correlates with later recurrence of the disease and with the formation of distant metastasis. Thymosin β 15 is more likely to be upregulated in invasive and metastatic cancers than in low-grade non-invasive cancers. Patients who have positive thymosin β 15 staining at the time of diagnosis are 7 times more likely to have tumor recurrence than patients who are thymosin β 15-negative. We are also able to detect thymosin β 15 in the urine of patients with prostate cancer. Thymosin β 15 levels drop after prostate removal surgery and rise again if there is local or distant recurrence of tumor growth. Our results therefore indicate that thymosin β 15 is useful both as a marker for future outcome in patients with newly diagnosed cancer and as a tool for follow-up of prostate cancer patients to determine if tumor recurrence takes place.

Dual functions for T β 15 in tumor progression: intracellular motility and extracellular angiogenic functions

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Beta-thymosins are a family of proteins (e.g. T\$15), which correlate with increasing tumor malignancy and metastatic potential. These proteins bind to Gactin, retard actin polymerization and influence cell motility. TB4 also acts as an extracellular factor, which promotes endothelial cell migration in vitro. We examined the β -thymosin profile in low and high metastatic variants derived from the same parent prostate carcinoma cell lines. Northern blot analysis of cells with increasing metastatic potential revealed changes in the B-thymosin composition characterized by rising levels of T β 10 and T β 15 with a simultaneous reduction in TB4. We asked if raising TB15 expression could enhance tumorgenesis, metastasis, or angiogenesis. Stable populations producing native TB15 (TB15wt) or a secreted form (secT β 15) were created from poorly metastatic variants of the Dunning carcinoma cell lines, which lack endogenous TB15. Western blot analysis revealed that both native T β 15wt and secT β 15 proteins were released from cells into culture medium. Conditions permitting the release of G-actin from cells, coupled with size-dependent fractionation of conditioned media, showed that extracellular T β 15 disassociated from the G-actin complex. This is consistent with the hypothesis that TB15 has intracellular and extracellular functions. In vitro growth rates of TB15wt, secTB15 and vector control cells were nearly identical, whereas subcutaneous tumors producing TB15 in Dunning rats exhibited a higher growth rate. After one month, tumors expressing TB15 were 50% larger than the vector controls, indicating that addition of TB15 accelerates tumor expansion. TB15 synthesis triggered a dramatic increase in cell motility, boosting serum response by 3 to 10 fold, relative to vector control. However, changes in cell motility did not translate into elevated rates of tumor metastasis, suggesting other factors may be required to alter this process. Further, synthetic protein and natural forms of TB15 obtained from conditioned medium acted as endothelial chemoattractants in vitro, yet did not stimulate the migration of human fibroblasts or smooth muscle cells. Together these results confirm that T β 15 is advantageous to tumor cells and points to a dual role in tumor progression: intracellular motility protein and extracellular angiogenic factor.



THYMOSIN BETA-15 PREDICTS FOR DISTANT FAILURE IN PATIENTS WITH CLINICALLY LOCALIZED PROSTATE CANCER—RESULTS FROM A PILOT STUDY

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ABSTRACT

Objectives. To report the results of a pilot study on the prognostic value of a newly identified actin-binding protein, thymosin beta-15 (T β 15), in predicting prostate-specific antigen (PSA) and bone failure in patients with Gleason 6/10 clinically localized prostate cancer.

Methods. Thirty-two patients (median age 70 years) with clinically localized, moderately differentiated (Gleason 6/10) prostate cancer treated by external beam radiotherapy alone (68.4 Gy) with available paraffin blocks at the Massachusetts General Hospital were evaluated for this pilot study. All patients had clinical Stage M0 disease at initial presentation, which was documented by bone scan (T1c-4,NX). Their corresponding biopsy specimens were stained immunohistochemically for T β 15, which was then correlated with the clinical outcome in a blinded manner. The median follow-up was 6 years (range 1 to 19) for all of the patients.

Results. The outcomes of the 32 patients can be grouped into three categories: patients with no evidence of disease (n = 11), patients with PSA failure without documented bone failure (n = 11), and patients with PSA failure and documented bone failure (n = 10). T β 15 staining intensity strongly correlated with clinical outcome. Of those patients whose specimens stained 3+ (strongest staining), 62% developed bone failure compared with 13% of those patients whose specimens stained 1+ (weakest staining) (*P* = 0.01). The 5-year freedom from PSA failure was only 25% for those patients with 3+ staining compared with 83% for those with 1+ staining (*P* = 0.02).

Conclusions. The results of this pilot study have demonstrated that T β 15 staining intensity may be a potentially important marker to identify high-risk patients with moderately differentiated, clinically localized prostate cancer. UROLOGY **55**: 635–638, 2000. © 2000, Elsevier Science Inc.

D espite aggressive radiotherapy, it is known that a substantial percentage of patients with clinical Stage M0 prostate cancer will have progression with distant metastases at 15 years.^{1–5} Many of these patients probably had occult micrometastatic disease at presentation. From a therapeutic standpoint, it is important at the time of the initial diag-

nosis to identify patients who are at high risk of harboring micrometastatic disease because systemic therapy has potential value in eradicating micrometastatic disease.^{6–8}

Gleason grade in its extremes does predict for distant metastases, but up to 80% of patients at their initial presentation have moderately differentiated tumors, which highlights the need for other prognostic markers in this group of patients. Cell motility is known to underlie many of the steps involved in the metastatic cascade. Bao *et al.*⁹ have identified an actin-binding protein, thymosin beta-15 (T β 15), as a potentially important marker for prostate cancer cells with high metastatic potential by enhancing cell motility.

A direct correlation between an increasing Gleason grade and positivity of staining has been re-

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ported.⁹ Interestingly, for patients with moderately differentiated tumors (Gleason 6-7), a wide range of staining intensities was observed, with specimens staining strongly, weakly, or not at all. This raises the possibility that T β 15 staining can identify which patients with moderately differentiated tumors are at greatest risk of harboring micrometastatic disease and, hence, at greatest risk of subsequent development of biochemical and/or clinically evident bone failure. As the vast majority of patients at initial presentation have moderately differentiated tumors, this is a clinically important question.

This is the first reported clinical study to our knowledge to determine, in a blinded manner, whether T β 15 staining of the initial biopsy specimen predicts for the subsequent development of biochemical and/or bone failure for patients with moderately differentiated prostate cancers who had clinical Stage M0 disease at the initial presentation. This study was designed to be a pilot study consisting of a relatively modest number of patients, which would lead to a larger scale multi-institutional study if a positive correlation were found.

MATERIAL AND METHODS

Three hundred patients with moderately differentiated prostate adenocarcinoma (Gleason grade 3 of 5 and score 6 of 10) treated by external beam radiation and with up-to-date follow-up data were identified from the Massachusetts General Hospital Radiation Oncology prostate cancer data base. Thirty-two of these patients had available paraffin blocks from the initial biopsy at Massachusetts General Hospital for analysis. The clinical staging was determined using the AJCC criteria, and all patients had Stage M0 disease at the initial presentation, as confirmed by bone scan. The distribution of patients by clinical T stage was as follows: T1c, 3; T2a, 2; T2b, 11; T3, 13; T4, 2; and TX, 1. The median pretreatment prostate-specific antigen (PSA) level was 7.1 ng/mL (range 1.0 to 49.9). The median age for all patients was 70 years (range 63 to 86). The median follow-up was 6 years (range 1 to 19). The patients were treated with external beam irradiation alone to a total dose of 68.4 Gy using high-energy photon irradiation. No systemic therapy, hormonal or chemotherapy, was rendered to these patients.

The corresponding tissue specimens from the initial biopsy (before treatment) were obtained for all 32 patients. All specimens were fixed in formalin and embedded in paraffin. On review by light microscope, all residual tissue contained moderately differentiated adenocarcinoma, Gleason score 6/10. Immunohistochemical staining was performed as previously described by the use of avidin-biotin-conjugated peroxidase complex technique (Vector Laboratories, Burlingame, Calif). T β 15 antibody was a generous gift from L. Bao and B. Zetter (Children's Hospital, Boston, Mass). Sections without primary antibody or sections with benign prostatic tissue were used as negative controls. Staining and interpretation of staining were done without knowledge of the clinical outcome.

RESULTS

The outcomes of these 32 patients can be grouped into three categories: patients with no ev-

idence of disease (n = 11), patients with PSA failure without documented bone failure (n = 11), and patients with PSA failure and documented bone failure (n = 10). PSA failure was defined as three consecutive post-treatment rises. Bone failure, when present, was documented by bone scan. Of the 11 patients with PSA failure without bony metastases, 5 had received hormonal therapy at the time of the initial PSA failure. Of the 10 patients with PSA failure and documented bone failure, only 1 had received hormonal therapy when the PSA elevation was first detected. Therefore, hormonal therapy likely delayed the appearance of distant metastases in these 5 patients with PSA elevation without evident bony metastases.

Normal prostatic acinar epithelium and benign hyperplastic epithelium stained negatively for T β 15 (Fig. 1A), confirming the observation made by Bao et al.⁹ Interestingly, endothelial cells within the biopsy specimens stained with moderate intensity for T β 15, which was comparable in all specimens. However, the invariability of the presence of blood vessels in the tissue and the consistent endothe lial staining with T β 15 antibody prompted us to use endothelial staining as a reliable positive internal control and a reference point for scoring tumor cell staining. When most of the tumor cells (greater than 70%) stained with lower intensity than the endothelial cells, the specimen was graded negative/weakly positive (1+) (Fig. 1B). When most of the tumor cells (greater than 70%) stained with equivalent intensity to the endothelial cells or had a mixed staining pattern, the specimen was scored as moderate staining (2+) (Fig. 1C). When most of the tumor cells (greater than 70%) stained more intensely than the endothelial cells, the specimen was graded as strongly positive (3+) (Fig. 1D).

A T β 15 stain of 3+ strongly predicted the subsequent development of PSA failure with bony metastases. For all 32 patients, 8 (62%) of the 13 patients with 3+ staining developed bony metastasis compared with only 1 (13%) of 8 patients with 1+ staining and 1 (14%) of 7 patients with 2+ staining (P = 0.01 by exact trend test).

At 5 years of follow-up, a T β 15 staining score of 3+ strongly predicted for PSA failure. For all 25 patients followed up for more than 5 years, the 5-year actuarial biochemical control rate was 44%. For a T β 15 stain of 1+, 2+, or 3+, the corresponding 5-year actuarial biochemical control rate was 83% (n = 6), 43% (n = 7), and 25% (n = 12), respectively (*P* = 0.02 by exact trend test). At 5 years, the positive predictive value for a T β 15 stain score of 3+ was 86% (with biochemical failure as the end point). The negative predictive value for a T β 15 stain score of 1+ was 71% (with freedom from biochemical failure as the end point).

To control for confounding by other predictors of



FIGURE 1. Immunohistochemical staining of $T\beta15$ on prostatic tissue. Affinity purified anti- $T\beta15$ antibody was used to stain (A) benign prostatic tissue and (B to D) Gleason grade 3/5 prostate adenocarcinoma. (A) Benign glandular cells (arrows) did not stain. In contrast, vascular endothelial cells (arrowheads) between the acini showed positive staining. (B) Acinar tumor cells (arrows) showed only weak focal staining and the endothelial cells (arrowheads) stained positively, indicating a true negative result. (C) Tumor cells (arrows) and endothelial cells (arrowheads) showed roughly equal staining intensity. (D) Tumor cells (arrows) show diffuse stronger staining compared with the endothelial cells (arrowheads).

failure, a multiple logistic regression model was fit to the data. Other covariates that were simultaneously put in the model were T stage, pretreatment PSA, age, and the subsequent development of local failure. The subsequent development of local failure (P = 0.34) and PSA (P = 0.59) were not significant predictors and were dropped from the final model. The final model included age (P = 0.18), T stage (P = 0.08), and staining intensity (P = 0.05). This analysis reveals that T β 15 staining predicted for PSA failure independent of the other factors. The adjusted odds ratio for staining was 4.35, with a 95% confidence interval of 1.01 to 18.86. Thus, for each unit increase in staining value (from 1 to 2 or 2 to 3), the odds of developing biochemical failure increase by an estimated factor of 4.35.

COMMENT

Metastases can occur quite early in the course of human prostate cancer. A recent study revealed

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that more than one half of patients with pathologically organ-confined disease had positive bone marrow specimens by reverse transcriptase-polymerase chain reaction analysis.¹⁰ These data, along with clinical observations, support the hypothesis that early dissemination of prostate cancer may occur despite local control of the disease.

Given the magnitude of this problem, molecular markers have been sought to identify high-risk patients. Low expression of cell cycle regulatory proteins such as p53, pRB, and p27 and high expression of Ki-67 have been found to predict an unfavorable outcome in patients with prostate cancer.^{11–13} It has been demonstrated that time-lapse videomicroscopic images can distinguish high from low metastatic potential cell lines when membrane ruffling, pseudopodal extension, and cellular translations of single cells are analyzed.¹⁴ Hence, enhanced cell motility, perhaps T β 15-dependent, underlies the metastatic process.

T β 15 mRNA is absent in most normal tissues, with the exception of the testes.¹⁵ Other tumor cell lines, including highly metastatic prostate, lung, breast, and melanoma cell lines, strongly express T β 15. Corresponding cell lines with low metastatic potential have low levels or absent T β 15 staining.

The wide range of T β 15 staining intensities among cases of moderately differentiated tumors raised the question of whether T β 15 staining can effectively discriminate between moderately differentiated tumors that are at high risk of harboring occult metastases from those at lower risk. The results from the 32 patients in this study with Gleason score 6/10 tumors reveal that at 5 years of follow-up, a T β 15 stain of 3+ has a high positive predictive value (86%) for the subsequent development of PSA failure. Likewise, at 5 years, a T β 15 stain of 1+ has a high negative predictive value (71%), with freedom from PSA failure as the end point.

The results of this pilot study suggest that T β 15 staining of the initial biopsy specimens from patients with Stage M0 moderately differentiated prostate adenocarcinoma has strong predictive value for the subsequent development of biochemical and clinically evident bone failure. Further investigations on a larger patient population are planned.

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DEPARTMENT OF THE ARMY US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND 504 SCOTT STREET FORT DETRICK, MARYLAND 21702-5012

REPLY TO ATTENTION OF:

MCMR-RMI-S (70-1y)

17 Jun 02

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for grants. Request the limited distribution statements for the Accession Documents listed at enclosure be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Μ Dep Chi ₽f of Staff for ormat ion Management

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