Prognostic Significance of Metastasis-Associated Protein S100A4 (Mts1) in Prostate Cancer Progression and Chemoprevention Regimens in an Autochthonous Mouse Model

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ABSTRACT

Purpose: **We recently showed that metastasis-promoting *Mts1* gene (S100A4) and protein is overexpressed during progression of prostate cancer in humans. The purpose of this study was to assess the expression of S100A4 during autochthonous prostate cancer progression in transgenic adenocarcinoma of the mouse prostate (TRAMP) model. Because oral consumption of green tea polyphenols (GTP) has been shown to inhibit metastasis and prostate cancer in TRAMP, we further assessed the significance of S100A4 during chemoprevention regimen.**

Experimental Design: **Male TRAMP mice 8 weeks of age were equally divided into two groups. A freshly prepared 0.1% GTP solution in tap water was supplied thrice a week to experimental animals as the sole source of drinking fluid for 24 weeks, whereas the control group of animals received the same tap water throughout the experiment. The animals were sacrificed at 0, 8, 16, and 24 weeks of GTP feeding and were analyzed for S100A4 and E-cadherin. Additional untreated and treated nontransgenic controls were also included in the study.**

Results: **With the progression of age and prostate cancer growth in TRAMP mice, an increase in the expression of S100A4 at mRNA and protein level in dorsolateral prostate, but not in nontransgenic mice, occurred. GTP feeding to TRAMP mice resulted in marked inhibition of prostate cancer progression, which was associated with reduction of S100A4 and restoration of E-cadherin.**

Conclusions: **S100A4 represents a promising marker for prostate cancer progression and could be employed as a biomarker in chemoprevention regimens.**

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INTRODUCTION

Prostate cancer is the most common visceral cancer diagnosed in men and is currently the second leading cause of cancer related deaths in the United States (1). A similar trend is found in the Western world (2–4). The lack of effective therapies for advanced prostate cancer reflects to a large extent paucity of knowledge about the molecular pathways involved in prostate cancer development. Thus, the identification of new predictive biomarkers will be important for improving clinical management, outcome of treatment protocols, and assessing the effectiveness of chemopreventive regimens, all of which could lead to improved survival of patients with prostate cancer. Molecular targets for prostate cancer, especially those that are indicative of invasiveness of the disease, will be excellent candidate targets for staging the disease and establishing effectiveness of chemoprevention of prostate cancer (5, 6).

S100A4 (also known as p9Ka and mts1), a member of the S100 calcium-binding protein family, has been associated with invasion and metastasis of cancer cells (7). S100A4 has been shown to be up-regulated in transformed cells and in many forms of cancer, including breast, ovary, thyroid, lung, esophageal squamous cell carcinoma, gastric, and colon (8). Recently, we showed that both S100A4 protein and mRNA are overexpressed during progression of prostate cancer in humans and suggested that it could be used as a potential biomarker for prostate cancer diagnosis and assessment of efficacy during clinical management (9). We now hypothesize that S100A4 protein could also be an excellent surrogate biomarker for establishing effective chemopreventive regimes for prostate cancer patients.

For relevance to humans, prostate cancer studies should be conducted in animal models where the disease occurs in a manner similar to that in humans. The transgenic adenocarcinoma of the mouse prostate (TRAMP) is one such model for prostate cancer in which progressive forms of the disease occurs in a manner very similar to the human disease (10). In this model, expression of the *SV40* early genes (T and t antigen, Tag) are driven by the prostate-specific promoter probasin that leads to cell transformation within the prostate (10). One hundred percent of male TRAMP mice develop prostate cancer without any chemical or hormonal treatment (11). Recent studies from our laboratory and elsewhere have established the utility of these mice for prostate cancer chemoprevention and intervention studies (12–16). Using the TRAMP model, we recently showed that oral consumption of green tea polyphenols (GTP), equivalent of six cups per day, results in significant inhibition in the development, progression, and metastasis of prostate cancer (16). In the present study, using tissues obtained from age-matched nontransgenic and TRAMP mice, we show that the expression of S100A4 mRNA and protein significantly increases during progressive stages of prostate cancer development and this increase in expression is abrogated by oral consumption of GTP. An inverse relation between

S100A4 and E-cadherin expression levels exists in many types of cancer such as breast, lung, and gastric cancer. We also observed that loss of E-cadherin protein in prostate tissue that occurred during the progression of prostate cancer in TRAMP mice was restored by GTP consumption. We suggest that S100A4 could be a candidate biomarker for prostate cancer development and for assessing effectiveness of chemopreventive regimens.

MATERIALS AND METHODS

Tissues used in this study were those derived from the archival samples of our previous study (16). Briefly, male and female TRAMP mice developed on a pure C57BL/6 background, heterozygous for the probasin-Tag transgene, were bred and maintained in the Animal Care Facility (Case Western Reserve University, Cleveland, OH). Transgenic males and the non-transgenic littermates were routinely obtained as [TRAMP C57BL/6] F_1. GTP (>95% enriched preparation) was obtained from Natural Resources & Products (Charlottesville, VA). Chromatographic analysis of this mixture showed that it contains four major polyphenolic constituents: epigallocatechin-3-gallate (62%), epicatechin-3-gallate (24%), epigallocatechin (5%), epicatechin (6%), and caffeine (~1%). A freshly prepared solution of 0.1% GTP in tap water was supplied every Monday, Wednesday, and Friday to experimental animals (GTP-fed group) as the sole source of drinking fluid for 24 weeks, whereas the control group of animals (water-fed group) was supplied with the same tap water throughout the experiment. The feeding protocol mimics an approximate consumption of six cups of green tea per day by an average adult human (16). Additional untreated and treated nontransgenic controls were also included in the study. At desired times during feeding regimens, the animals from both experimental and control groups were killed and the dorsolateral prostate was carefully removed under the microscope for further studies. All experiments were conducted using the highest standards for animal care in accordance with the NIH guidelines for the Care and Use of Laboratory Animals.

Western Blot Analysis. The total cell lysate was prepared in cold lysis buffer [50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EGTA, 1 mmol/L EDTA, 20 mmol/L NaF, 100 mmol/L Na_3VO_4, 0.5% NP40, 1% Triton X-100, and 1 mmol/L phenylmethylsulfonyl fluoride (pH 7.4)] with freshly added protease inhibitor cocktail (Protease Inhibitor Cocktail Set III, Calbiochem, La Jolla, CA). The lysate was collected in a microfuge tube and was cleared by centrifugation at 14,000 × *g* for 15 minutes at 4°C. The supernatant was aliquoted, and stored at −80°C. The protein content in the lysates was measured by bicinchoninic acid protein assay (Pierce, Rockford, IL) as per the manufacturer's protocol.

For Western blot analysis, 40 μg of protein were resolved over 18% (for S100A4) and 12% (for E-cadherin) Tris-glycine polyacrylamide gels (Novex, Carlsbad, CA) and then transferred onto the nitrocellulose membranes. The blots were blocked in blocking buffer (5% nonfat dry milk) for 2 hours at room temperature followed by incubation with appropriate primary monoclonal antibody (human reactive S100A4 and E-cadherin obtained from Dako Corp., Santa Barbara, CA and Santa Cruz Biotechnology, Santa Cruz, CA, respectively) in blocking buffer for 3 hours at room temperature. After a brief washing, the blots were incubated with appropriate secondary antibody horseradish peroxidase conjugate (Amersham Biosciences, Piscataway, NJ) and detected by enhanced chemiluminescence (Amersham Biosciences) and autoradiography using XAR-5 film (Eastman Kodak, Rochester, NY).

Immunohistochemistry. Paraffin-embedded sections (4 μ mol/L) from dorsolateral prostate of nontransgenic and TRAMP mice were deparaffinized, rehydrated, and immersed in 0.3% H_2O_2 for 20 minutes to block endogenous peroxidase activity. The sections were incubated in primary antibodies of S100A4 and E-cadherin, at 1:200 dilution overnight at 4°C. Control sections were incubated with antisera in the presence of 10-fold excess of immunoglobulin G normal goat serum. After washing thrice in TBS, sections were incubated for 2 hours at room temperature with appropriate horseradish peroxidase–conjugated secondary antibody. Immunoreactive complexes were detected using 3,3′-diaminobenzidene (Dako). Slides were then counterstained in hemotoxylin, mounted in crystal mount, and coverslipped in 50:50 xylene/Permount as described previously (16). Sections were visualized on a Zeiss-Axiophot DM HT microscope. Images were captured with an attached camera linked to a computer.

Total RNA Isolation and cDNA Synthesis. Total RNA was isolated from dorsolateral prostate of mice using the Trizol reagent (Life Technologies, Gaithersburg, MD) and was checked for its purity and concentration as previously described (9). Two micrograms of total RNA were reverse transcribed using murine MLV-reverse transcriptase and oligodeoxythymidylate primer from Promega (Madison, WI) for cDNA synthesis.

Semiquantitative PCR of S100A4. Two microliters of the cDNA were subjected to PCR in PCR buffer [20 mmol/L Tris-HCl (pH 8.4) and 50 mmol/L KCl], 1.5 mmol/L $MgCl_2$, 0.2 mmol/L deoxynucleotide triphosphate, 2.5 units of Platinum Taq DNA polymerase, and 0.2 μmol/L of each primer for S100A4 (forward 5′-TCAGAACTAAAGGAGCTGCTGACC-3′ and reverse 5′-TTTCTTCCTGGGCTGCTTATCTGG-3′) obtained from Promega in a total volume of 25 μL. The cDNA was amplified with an initial denaturation at 94°C for 2 minutes followed by the sequential cycles of denaturation at 94°C for 45 seconds, annealing at 59°C for 45 seconds, and extension at 72°C for 1 minute for 30 cycles, with final extension at 72°C for 10 minutes. A constitutively expressed *GAPDH* gene (forward 5′-TGAAGGTCGGAGTCAAGCGATTTGGT-3′ and reverse 5′-CATGTGGGCCATGAGGTCCACCAC-3′) was coamplified to confirm the equal loading of the cDNA. Fifteen-microliter aliquots of PCR products were electrophoresed on 1.5% agarose gel and visualized by ethidium bromide staining under UV transilluminator.

Densitometric Analysis. Immunoblots were scanned by HPK Precisionscan Pro 3.13 (Hewlett-Packard Co., Palo Alto, CA). Densitometry measurements of the scanned bands were done using digitalized scientific software program UN-SCAN-IT (Silk Scientific Co., Orem, UT). Data were normalized to β-actin and expressed as mean ± SE of three separate set of experiments followed by appropriate statistical analysis.

Statistical Analysis. Results were analyzed using a two-tailed Student's *t* test to assess statistical significance between

groups. To assess the change in expression of S100A4 in water-fed TRAMP mice, comparisons were made with the water-fed animals of the preceding age. For analysis of the effect of oral feeding of GTP, comparisons were made with age-matched water-fed TRAMP mice. $P < 0.05$ was considered statistically significant.

RESULTS

S100A4 Expression in Nontransgenic and TRAMP Mice. Earlier, we have shown that the expression of S100A4 protein in prostate significantly increases with progression of prostate cancer in human patients (9). As a first attempt towards identifying the expression of S100A4 during prostate cancer progression in TRAMP mouse, we did immunoblot analysis for this protein in dorsolateral prostate obtained from 8 weeks (no cancer), 16 weeks (well differentiated with multiple epithelial mitotic figures and apoptotic bodies, invasive glands with stromal hypercellularity), 24 weeks (moderately differentiated with many acini completely filled with tumor yet still forming some glandular structures), and 32 weeks (poorly differentiated with sheets of malignant cells with little or no gland formation) old TRAMP mice. We also determined the expression of S100A4 in the dorsolateral prostate of age-matched nontransgenic littermates. As shown in Fig. 1, S100A4 protein expression increased gradually in dorsolateral prostate of TRAMP with increasing age and cancer development. This age-dependent increase was not observed in nontransgenic mice. As evident from the results of densitometric analysis, the expression of S100A4 protein showed an increase of 250% at 16 weeks, 340% at 24 weeks, and 525% at 32 weeks as compared with age-matched nontransgenic mice (Fig. 1*A*). There was no significant increase of S100A4 protein expression in dorsolateral prostate of transgenic mice of 8 weeks as compared with the age-matched nontransgenic control (Fig. 1*A*). A similar increasing trend was found for S100A4 mRNA levels (Fig. 2). As shown in Fig. 2, the level of S100A4 mRNA was significantly higher in high-grade cancer tissue compared with low-grade cancer and nontransgenic mice.

We next examined the protein expression of S100A4 by immunohistochemistry in tissues obtained from nontransgenic

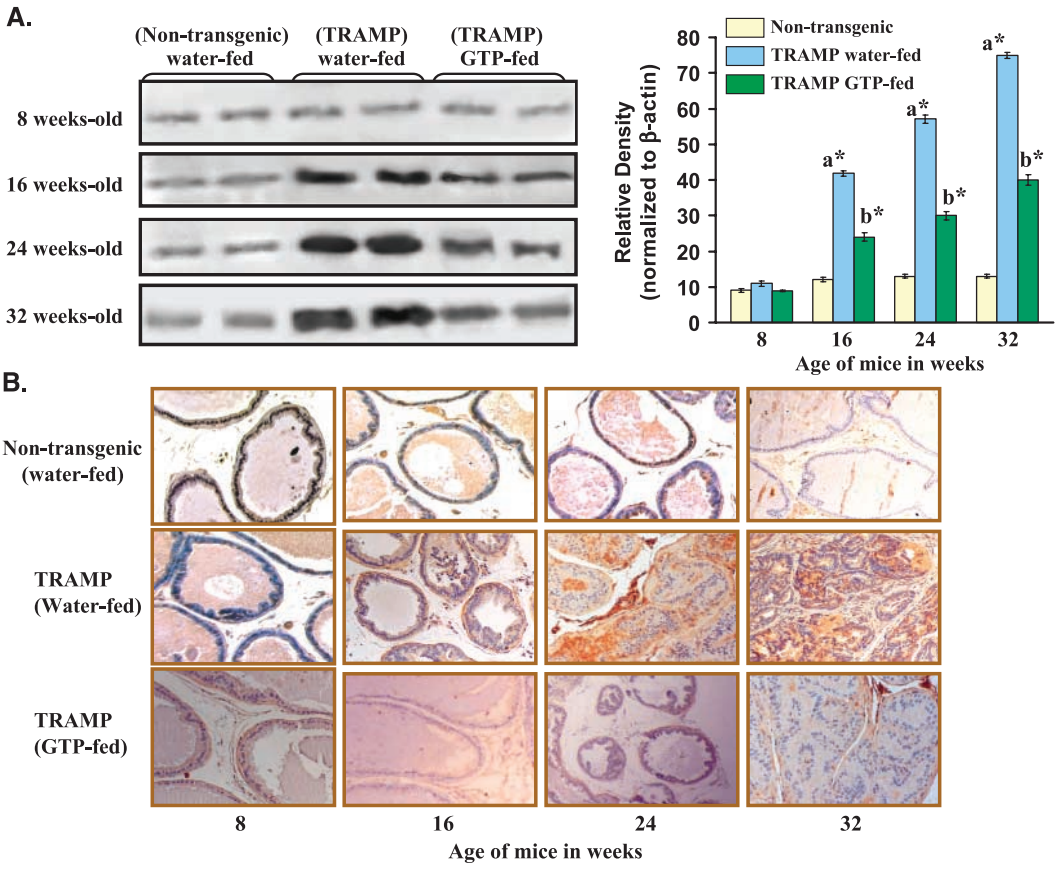


Fig. 1 Expression of S100A4 protein in dorsolateral prostate during progressive stages of prostate cancer development in TRAMP mice: effect of oral consumption of GTP. *A,* Immunoblot analysis of S100A4 in the dorsolateral prostate of 8-, 16-, 24-, and 32-week-old nontransgenic, water-fed and GTP-fed TRAMP mice. Equal loading of protein was confirmed by stripping the blots and reprobing with β-actin antibody. *Histogram,* relative density of the bands normalized to β-actin. Representative data for two mice per group. Similar trend in additional four mice. *Columns,* means of relative densities; *bars,* ± SE. (*a*) data compared with water-fed animals of the preceding age, (*b*) data compared with age-matched water-fed animals. *, $P <$ 0.001. Details in Materials and Methods. *B,* representative photomicrographs (magnification ×40) of immunohistochemical staining for S100A4 in the dorsolateral prostate of 8-, 16-, 24-, and 32-week-old nontransgenic, water-fed TRAMP and GTP-fed TRAMP mice. The immunostaining data was confirmed in two or more slides from six or more animals of each group.

and TRAMP mice. A progressive increase in the protein expression of S100A4 was observed in the dorsolateral prostate tissue of TRAMP mice as cancer progressed from 8 weeks (no cancer) to well-differentiated adenocarcinoma at 16 weeks of age to poorly differentiated adenocarcinoma at 32 weeks of age, suggesting that S100A4 expression was dependent on tumor grade. Foci of intense staining were observed in all tumor sections and malignant epithelial cells were highly positive for S100A4. In particular, S100A4 expression was faint in the cytoplasm of the nontransgenic specimens at all ages and in TRAMP mice of 8 weeks age (Fig. 1*B*).

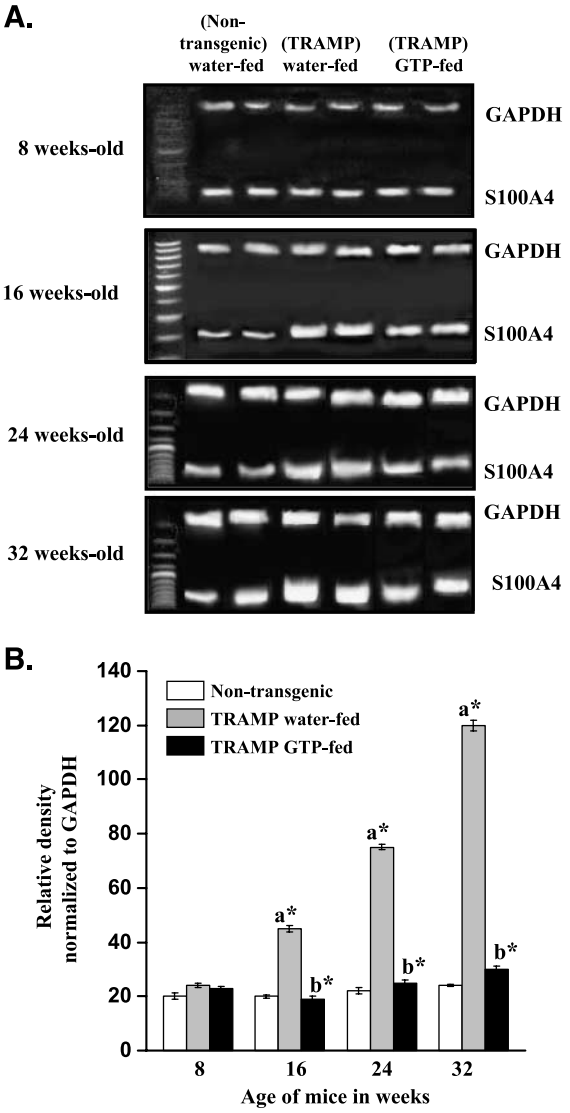


Fig. 2 Expression of S100A4 mRNA in dorsolateral prostate during progressive stages of prostate cancer development in TRAMP mice: effect of oral consumption of GTP. *A,* reverse transcription-PCR analysis of S100A4 in the dorsolateral prostate of 8-, 16-, 24-, and 32-week-old nontransgenic, water-fed and GTP-fed TRAMP mice. Equal loading of reverse transcription-PCR product was confirmed by GAPDH as an internal control. *B, histogram,* relative density of bands normalized to GAPDH. Representative data for two mice per group. Similar trend in additional four mice. *Columns,* means of relative densities; *bars,* ± SE. (*a*) data compared with water-fed animals of the preceding age, (*b*) data compared with age-matched water-fed animals. *, $P < 0.001$. Details in Materials and Methods.

Effect of GTP Consumption on S100A4 Expression in TRAMP Mice. TRAMP mice fed with GTP showed a significant reduction in S100A4 protein expression in the dorsolateral prostate as compared with the water-fed TRAMP group (Fig. 1*A*). The densitometric analysis of the bands (normalized to β-actin) showed a significant decrease ($P < 0.001$) in S100A4 protein expression (40-50%) in GTP-fed TRAMP mice as compared with age-matched water-fed TRAMP mice (Fig. 1*A*). GTP supplementation to the nontransgenic littermates did not cause any significant alteration in the protein expression of S100A4 in the dorsolateral prostate (data not shown). Similar results were observed when the mRNA expression of S100A4 was assessed (Fig. 2). GTP-fed TRAMP mice exhibited a significant ($P < 0.001$) reduction in the expression of S100A4 mRNA (50-65%) as compared to that in age-matched water-fed TRAMP mice (Fig. 2).

In TRAMP mice, an extensive S100A4 staining was observed in the lumen region of the epithelium with the progression of the disease from 16 to 32 weeks. GTP feeding resulted in the marked reduction in S100A4 staining in TRAMP mice as compared with water-fed TRAMP mice (Fig. 3). Both control and GTP-fed nontransgenic animals exhibited a faint S100A4 expression (data not shown).

Effect of GTP Consumption on E-cadherin Protein Expression in TRAMP Mice. S100A4 expression has been shown to be inversely correlated with the loss of expression of various cytoskeleton molecules such as E-cadherin and catenin, during the metastasis of various types of cancers (17, 18). We have earlier shown that the normal pattern of E-cadherin is lost during prostate cancer progression in TRAMP mice in a fashion that parallels human prostate cancer (14). Therefore, we evaluated the effect of GTP consumption by TRAMP on the levels of E-cadherin. As shown in Fig. 3, GTP feeding to TRAMP mice for 24 weeks was effective in significant restoration (up to 80%, $P < 0.001$) of the protein expression of E-cadherin in the dorsolateral prostate (Fig. 3). The immunohistochemical analysis of the dorsolateral prostate in 32-week-old GTP-fed TRAMP mice showed a marked restoration E-cadherin in the basement membrane compared with the water-fed TRAMP mice (Fig. 3*B*). No significant alteration in the E-cadherin protein expression was observed in the GTP-fed nontransgenic littermates when compared with the corresponding control (data not shown).

The combined analysis of E-cadherin and S100A4 has been suggested as a good prognostic indicator of patients with several types of cancers and that tumors with overexpression of S100A4 and reduced E-cadherin could be classified as highly malignant phenotype (17, 18). Therefore, we analyzed the results in terms of ratio between S100A4 and E-cadherin expression following GTP feeding during progressive stages of cancer development in TRAMP mice. We observed a significant shift favoring S100A4 expression as a function of age and disease progression in transgenic water-fed mice. However, the balance between S100A4 and E-cadherin was restored in mice that received oral infusion of GTP (Fig. 4).

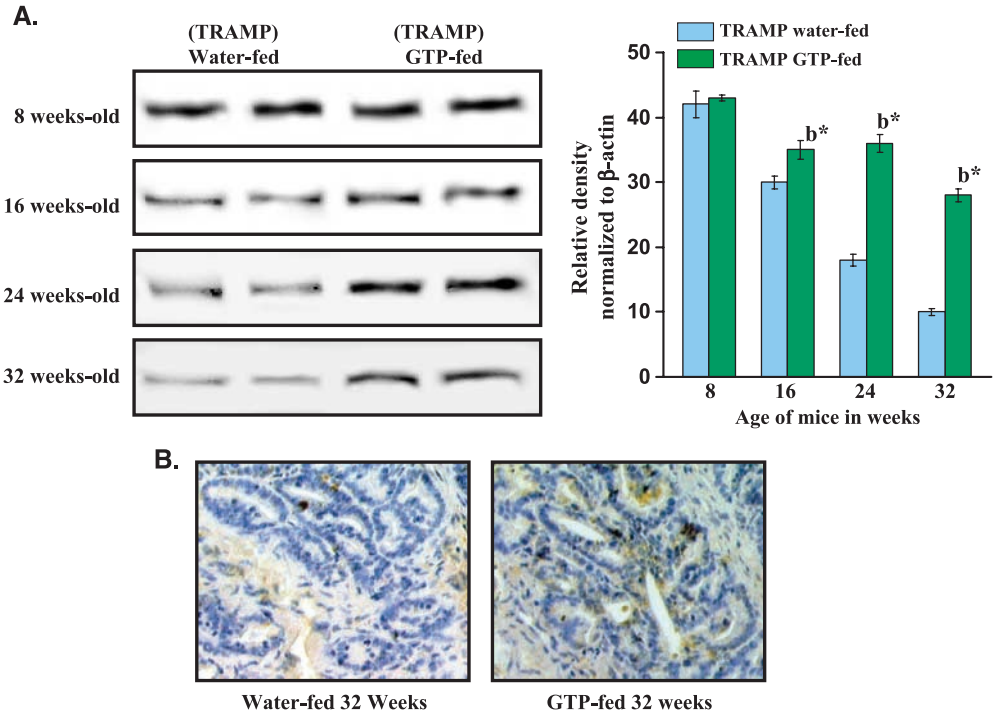
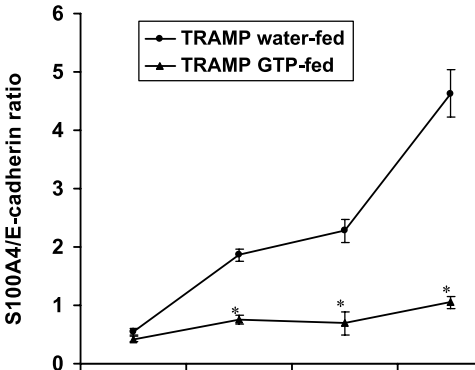


Fig. 3 Expression of E-cadherin protein in dorsolateral prostate during progressive stages of prostate cancer development in TRAMP mice; effect of oral consumption of GTP. *A,* immunoblot analysis of E-cadherin in the dorsolateral prostate of 8-, 16-, 24-, and 32-week-old water-fed and GTP-fed TRAMP mice. Equal loading of protein was confirmed by stripping the blots and reprobing with β-actin antibody. *Histogram,* relative density of the bands normalized to β-actin. Representative data for two mice per group. Similar trend in additional four mice. *Columns,* means of relative densities; *bars,* ± SE. (*b*) data compared with age-matched water-fed animals. *, $P < 0.001$. Details in Materials and Methods. *B,* Representative photomicrographs (magnification ×40) of immunohistochemical staining for E-cadherin in the dorsolateral prostate of a 32-week-old water-fed TRAMP and GTP-fed TRAMP mice. The immunostaining data was confirmed in two or more slides from six or more animals of each group.

DISCUSSION

One important consideration towards the intervention and prevention of prostate cancer is the development of surrogate end point biomarker(s) that can be correlated with staging of disease along the course of tumor development. The important finding from our study is that the expression of S100A4 both at protein and mRNA level significantly increases with progressive stages of prostate cancer development in TRAMP, suggesting that this autochthonous model of prostate cancer development is ideal for detailed investigation into the role of S100A4 in prostate cancer development and metastasis. Based on these observations, we suggest that S100A4 could be employed as a predictive biomarker in prostate cancer development.

S100A4 represents a family of calcium binding proteins and as many as 17 such proteins have been identified in humans (7, 8, 19). Knowledge of the biological functions of calcium binding S100 proteins is limited, but some of them have been postulated to participate in signal transduction pathways regulating cell cycle progression and differentiation (8, 19). A member of S100 family of proteins is S100A4 (also known as p9Ka and Mts1), which acts in cell cycle progression, cell motility and modulates intercellular adhesion and invasive properties (20, 21). The *S100A4* gene has been linked to invasion and metastasis of cancer cells and has been shown to be up-regulated in a number of human cancers (21, 22). Based on recent studies, it is being increasingly appreciated that S100A4 protein could be developed as a biomarker for assessing stages and invasiveness of certain cancers. Studies have shown that breast cancers expressing high levels of S100A4 have a significantly worse



	8 weeks-old	16 weeks-old	24 weeks-old	32 weeks-old
TRAMP water-fed	0.55	1.86	2.28	4.63
TRAMP GTP-fed	0.42	0.75	0.69	1.05

Fig. 4 Effect of oral consumption of GTP on the ratio between S100A4 and E-cadherin expression in the prostate of TRAMP: the graph and table of the ratio between S100A4 and E-cadherin calculated from their respective relative densities normalized to β-actin. *Points,* ratios (S100A4/E-cadherin) of mean of relative densities; *bars,* ± SE. *, $P < 0.001$ compared with age-matched water-fed TRAMP mice.

prognosis than breast cancers negative for S100A4 (23, 24). Another recent study has shown that overexpression of S100A4 may be sufficient to induce a metastatic phenotype in human mammary tumor cells (25). Studies have also shown that S100A4 exerts its metastatic effect by facilitating extracellular matrix destruction. S100A4 also interacts with the sarcoplasmic reticulum and with actin stress fibers in a Ca^{2+}-dependent manner, resulting in the regulation of cell deformability and morphology (26). Additional studies indicated that overexpression of S100A4 correlates with the *in vitro* invasive potential of glioma cells and breast cancer conferring enhanced metastatic ability (25–27). Recently, we have shown that the protein level of S100A4 was significantly higher in human prostate cancer cell lines (22Rv1, DU-145, LNCaP, and PC-3) compared with normal human prostate epithelial and virally transformed prostate epithelial cells (9). The mRNA and protein level of S100A4 was significantly higher in high-grade cancer specimens compared with benign prostate hyperplasia, prostatitis, and low-grade cancer. The high levels of S100A4 observed in cancer tissue correlated with increasing tumor grade. In light of these facts, here we evaluated the efficacy of S100A4 as a potential prognostic biomarker during progressive stages of prostate cancer development in TRAMP mice. The expression of S100A4 protein was found to be significantly higher in dorsolateral prostate of TRAMP at all stages of tumor development compared to nontransgenic littermates where prostate cancer does not develop. These results provide, for the first time, evidence that increased expression of S100A4 may be indicative of cancer progression in TRAMP, a model in which prostate cancer progresses in a fashion similar to humans.

It is also desirable that such biomarkers should be able to monitor the efficacy of chemoprevention regimens. Our knowledge base for such biomarkers is limited and that has hindered drug development and chemopreventive regimens for prostate cancer. Therefore, the second major objective of our study was to determine whether S100A4 could be used as a biomarker for evaluating the efficacy of chemopreventive regimens. Since in our previous study, oral consumption of GTP by TRAMP was found to result in significant inhibition of prostate cancer development, here we determined mRNA and protein levels of S100A4 in archival samples of our previous study during prostate cancer chemoprevention by GTP (16). We found that oral feeding of GTP to TRAMP mice significantly reduced the mRNA and protein expression of S100A4 suggesting that S100A4 expression could be employed as a biomarker for prostate cancer chemoprevention. Much additional work is required to validate this suggestion.

Invasion of prostate cancer to distant sites causes metastatic disease that is regarded as the major cause of prostate cancer–related deaths in humans (28). Tumor cells acquire this increased invasive potential by a complex pathway, which include decreased cell substrate attachment and cell-cell adhesion as well as increased cell motility. E-cadherin has an important role in the homophilic cell-cell adhesion and is called an invasion suppressor gene (18, 29). Many studies have shown that the cadherin-catenin complex is correlated with the loss of cellular differentiation and acquisition of invasive and metastatic potential in human tumors, including head and neck, breast bladder, gastric, prostate, colon, and basal cell carcinoma of the skin (30–32). In the TRAMP model, loss of E-cadherin has been suggested to play a major role in modulating metastasis (33). It has been shown that S100A4 expression is inversely correlated with the expression of E-cadherin in various types of cancers (30–32). We have earlier shown that E-cadherin expression is lost during the progression of prostate cancer development in TRAMP mice (14). In view of the fact that S100A4 is inversely correlated with the expression of E-cadherin, we investigated whether the down-regulation of S100A4 protein by the oral feeding of GTP to TRAMP mice could reverse the levels of E-cadherin. We observed that GTP feeding to TRAMP mice caused a significant restoration of E-cadherin. These data suggest that the ratio of S100A4 to E-cadherin could be used as a potential predictive indicator for prostate cancer development and establishing efficacy of chemopreventive regimens.

We conclude that S100A4 represents a promising marker for prostate cancer progression and could be employed as a biomarker in prostate cancer chemoprevention regimens.

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