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## Green tea catechins suppress the DNA synthesis marker MCM7 in the TRAMP model of prostate cancer

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### ABSTRACT

Green tea catechins (GTCs) exert chemopreventive effects in many cancer models. Several studies implicate the DNA synthesis marker minichromosome maintenance protein 7 (MCM7) in prostate cancer progression, growth and invasion; representing a novel therapeutic target. In this study, we investigated the effect of GTCs on MCM7 expression in the transgenic adenocarcinoma mouse prostate model (TRAMP). DNA microarray, immunohistochemistry and Western blot analysis showed that GTCs significantly suppressed MCM7 in the TRAMP mice treated with GTCs. Our study indicates that the cellular DNA replication factor MCM7 is involved in prostate cancer (CaP) and MCM7 gene expression was reduced by GTCs. Together, these results suggest a possible role of GTCs in CaP chemoprevention in which MCM7 plays a critical role.

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## 1. Introduction

Prostate cancer (CaP) is currently recognized as the most frequent form of cancer in American men (Luo et al., 2002). According to the American Cancer Society, approximately 234 000 men will be diagnosed with CaP in the United States alone this year, and 27 350 men will die of the disease (Jemal

et al., 2006). The molecular events responsible for the progression of prostate cancer from a relatively indolent disease to one that can be life threatening are unclear (Luo et al., 2002). Since CaP is associated with a high morbidity and mortality rate (when the disease is clinically significant) and a long latency phase of 20–40 years, it represents an attractive target for chemoprevention (Siddiqui et al., 2006). A modest delay

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in the progression of this type of cancer chemoprevention could result in a substantial reduction in the incidence of the disease and improve the quality of life (Saleem et al., 2003).

Green tea, a popular beverage consumed worldwide, has been known for some time to have protective effects against some common types of cancer (Adhami et al., 2003; Doss et al., 2005; Stuart et al., 2006; Kumar et al., 2007). Epidemiological studies have indicated that Asian populations that consume tea on a regular basis have the lowest incidence of all types of cancer including CaP as compared to their western counterparts (Jian et al., 2004; Sim and Cheng, 2005; Siddiqui et al., 2006). The chemopreventive effects of green tea appear to be mediated by the polyphenolic compounds known as catechins. The main catechins found in green tea are (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin-3 gallate (ECG), and (-)-epigallocatechin-3-gallate (EGCG). Among these, EGCG is the most abundant catechin that has gained the most attention with respect to anticarcinogenic activity (Doss et al., 2005).

The TRAMP mouse model is a well-known autochthonous transgenic animal model of CaP that was developed as a very important tool for understanding the earlier events in the progression of this disease (Greenberg et al., 1995; Kaplan-Lefko et al., 2003). TRAMP mice express SV40 early genes (T and t antigen, Tag) under the control of the prostate-specific rat probasin promoter that leads to cell transformation within the prostate; mimicking the entire spectrum of human CaP progression (Greenberg et al., 1995; Kaplan-Lefko et al., 2003). The earliest pathology is prostatic intraepithelial neoplasia (PIN) as early as 12 weeks of age when expression of the transgene is maximal. Over the next 6-week period TRAMP mice commonly display progressive forms of adenocarcinoma and will ultimately develop poorly differentiated carcinoma by the time they reach 24–30 weeks of age (Greenberg et al., 1995; Kaplan-Lefko et al., 2003). Recent laboratory studies have indicated that green tea treatment can inhibit CaP development in this model (Gupta et al., 2001; Caporali et al., 2004).

In this study, we used the TRAMP model to compare time-course changes of gene expression in the prostate of wild-type (WT, non-transgenic), TRAMP water-fed and TRAMP mice treated with GTCs. Using the Affymetrix Mouse Genome Gene Chip, minichromosome maintenance protein 7 (MCM7) was identified as differentially overexpressed in TRAMP water-fed mice at the 17 week time point compared to WT (non-transgenic) mice. MCM7 expression levels continued to notably increase in the TRAMP water-fed mice as CaP progressed into poorly differentiated adenocarcinoma at 24 weeks of age. MCM7 is a key component of the DNA helicase complex with a critical role in controlling DNA replication (Labib and Diffley, 2001; Stoeber et al., 2001; Lei, 2005). Recent studies have indicated that MCM7 is associated with the aggressive phenotype of CaP; amplification and overexpression was notably linked to relapse and metastasis (Padmanabhan et al., 2004; Honeycutt et al., 2006; Ren et al., 2006). Since, MCM7 is believed to have an active role in CaP tumorigenesis; we investigated the effect of GTCs on MCM7 expression in the TRAMP mice. We have shown for the first time that GTCs suppresses a gene that is associated with prostate cancer progression, growth and tumor invasion.

## 2. Results

### 2.1. Effect of green tea on tumor development

In order to evaluate a possible GTC chemopreventive effect, TRAMP and WT (non-transgenic) mice 8 weeks of age were fed a solution of 0.3% GTC in tap water or tap water alone (controls) as drinking fluid. At 24 weeks of age, about 80% of GTC treated mice did not develop palpable tumor. However, 20% of TRAMP mice that were treated with GTCs did not respond to treatment showed palpable and fully developed tumors at 24 weeks of age. All tumors were confirmed by histology. Thus, GTC chemopreventive activity was evident in as many as 80% of the transgenic animals. These results fully confirm the data obtained previously by our group (Caporali et al., 2004).

### 2.2. Time course changes in gene expression in the prostate of WT and TRAMP mice

Figure 1 shows a representative heat map of a small group of early differentiated genes that were downregulated in the dorsolateral prostates of WT (non-transgenic) mice and upregulated in the TRAMP water-fed mice at the 12 and 17 week time point. Several of these genes were selected from the Affymetrix microarray analysis because they were upregulated (>2.2-fold) in the TRAMP water-fed mice compared to WT mice at 17 weeks of age. Among the list of genes, further DNA microarray analysis indicated that MCM7 expression levels continued to increase (>11.8-fold) in the TRAMP

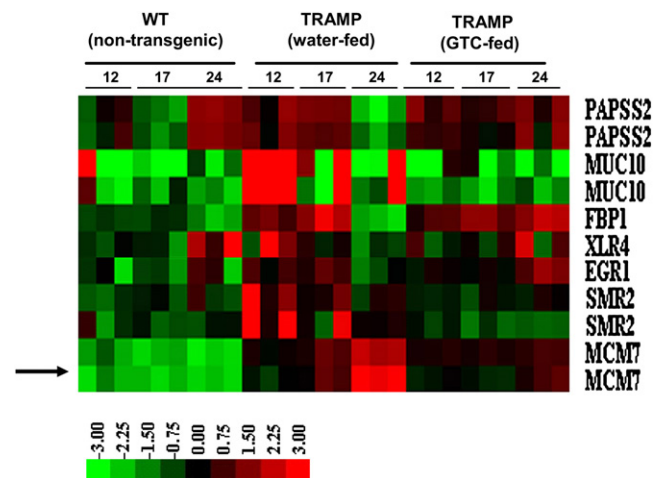


Figure 1 – Gene expression changes in the prostates of WT (non-transgenic), TRAMP water-fed and TRAMP mice treated with GTCs. Gene expression values were normalized,  $\log_2$ -transformed, and mean-centered to produce relative values. Each column represents pooled samples from three different prostate glands (dorsolateral) taken from WT, TRAMP water-fed and TRAMP treated with GTCs at the 12, 17, and 24 week time point. Some genes have multiple Affymetrix probe sets. Genes that are upregulated appear in red, and those that are downregulated appear in green. A color bar (bottom) relates to the intensity of the differences in gene expression.



water-fed mice at the 24-week time point compared to the WT mice. MCM7 expression increased during tumor progression in the TRAMP water-fed mice from the 12–24 week time point, and treatment with GTCs dramatically decreased MCM7 expression starting at 12 weeks of age (Figure 1).

### 2.3. MCM7 protein expression is increased in prostate cancer and GTC treatment suppresses MCM7 expression

Figure 2 shows immunohistochemical analysis of prostate tissue sections for MCM7 in WT (non-transgenic), TRAMP water-fed, and TRAMP mice treated with GTCs. The dorsolateral prostate of WT mice, composed of a single layer of epithelial cells and were rarely positive for MCM7 staining (Figure 2A).

A progressive increase in MCM7 protein expression was observed in TRAMP water-fed mice during prostate cancer progression. High grade PIN was observed in 12 week TRAMP mice; the cells exhibiting MCM7 staining were confined to the basal layer of the prostate epithelium (Figure 2B). By the time TRAMP mice reach the age of 17 weeks, PIN lesions progress to well differentiated prostate cancer. In this stage, epithelial cells invading through fibromuscular stromal layer were positive for MCM7 staining (Figure 2C). As shown in Figure 2D, the majority of cells in poorly differentiated CaP express MCM7 in TRAMP water-fed mice at 24 weeks of age. IHC indicated a marked reduction of MCM7 signal on GTC-fed TRAMP mice as compared to the TRAMP water-fed mice at 17 and 24 weeks (Figures 2C, D and E, F). In particular, in

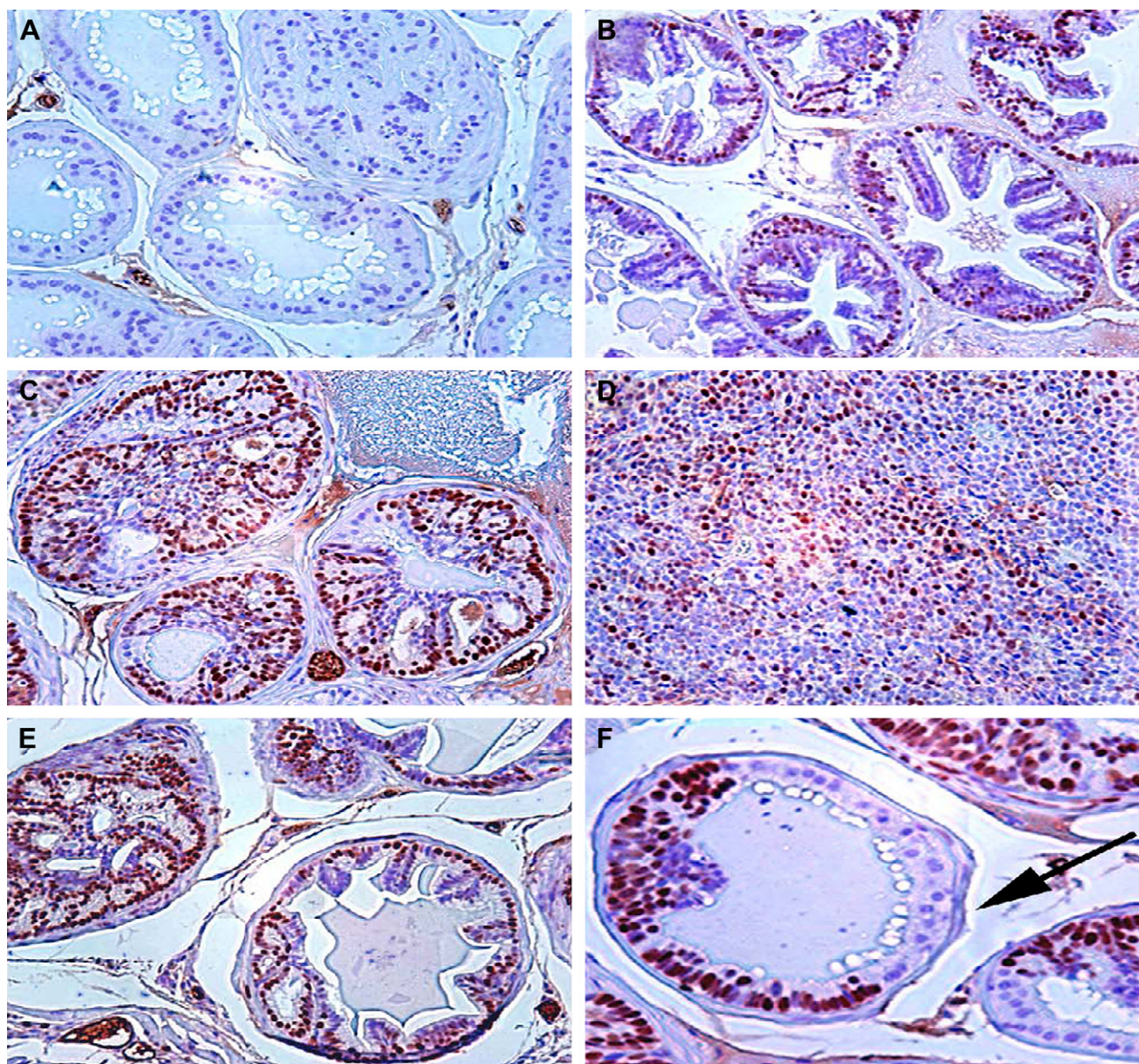


Figure 2 – Immunohistochemical staining for MCM7 protein in the dorsolateral prostate of WT and TRAMP mice. (A) WT (non transgenic) mouse at 24 weeks of age (20 $\times$ ); (B) TRAMP water-fed mouse at 12 weeks of age (20 $\times$ ); (C) TRAMP water-fed mouse at 17 weeks of age (20 $\times$ ); (D) poorly differentiated prostate cancer in TRAMP water-fed mouse at 24 weeks of age (20 $\times$ ); (E) prostate section of a GTCs-fed TRAMP mouse at 17 weeks of age (20 $\times$ ); (F) prostate section of a GTCs-fed TRAMP mouse at 24 weeks of age. MCM7 is not detectable in the normal monolayer of epithelial cells of the gland (arrow) but specifically expressed in the multilayer and high grade PIN lesions (40 $\times$ ).



Figure 2F (arrow) only high-grade PIN lesions were MCM7 positive, while normal monolayer cells within the same gland were negative to MCM7 staining. Parallel analysis using the proliferation marker, Ki-67 was also performed on contiguous sections of WT and TRAMP prostates at different stages of tumor development (Figures 3A–F). Both, MCM7 and Ki-67 showed close similarity in the pattern of expression. Figure 4 shows the percentage of positive MCM7 and Ki-67 cells in the prostates of WT and TRAMP mice with or without GTC treatment at 12, 17, and 24 weeks. MCM7 was significantly downregulated in the TRAMP treated with GTCs at 17 and 24 weeks compared to TRAMP water-fed ( $P < 0.001$ ). Western blot analysis further validated the loss of MCM7 expression

in the GTC-fed TRAMP mice (Figure 5A). The signal of MCM7 protein was only detectable at 17 weeks and this protein accumulated at very high levels in the prostates of TRAMP mice at 24 weeks (Figure 5A). The results from the densitometric analysis showed a significant ( $P < 0.001$ ) increase in MCM7 protein expression in the TRAMP water-fed mice at 24 weeks compared to the TRAMP water-fed mice at 17 weeks (Figure 5B). In our experimental conditions, MCM7 was undetectable at 12, 17, and 24 weeks in TRAMP mice receiving GTCs (Figure 5A). Densitometric analysis revealed that GTC-fed TRAMP mice showed a significant ( $P < 0.001$ ) decrease in MCM7 protein expression as compared to age-matched TRAMP water-fed mice (Figure 5B). Thus, it appears that

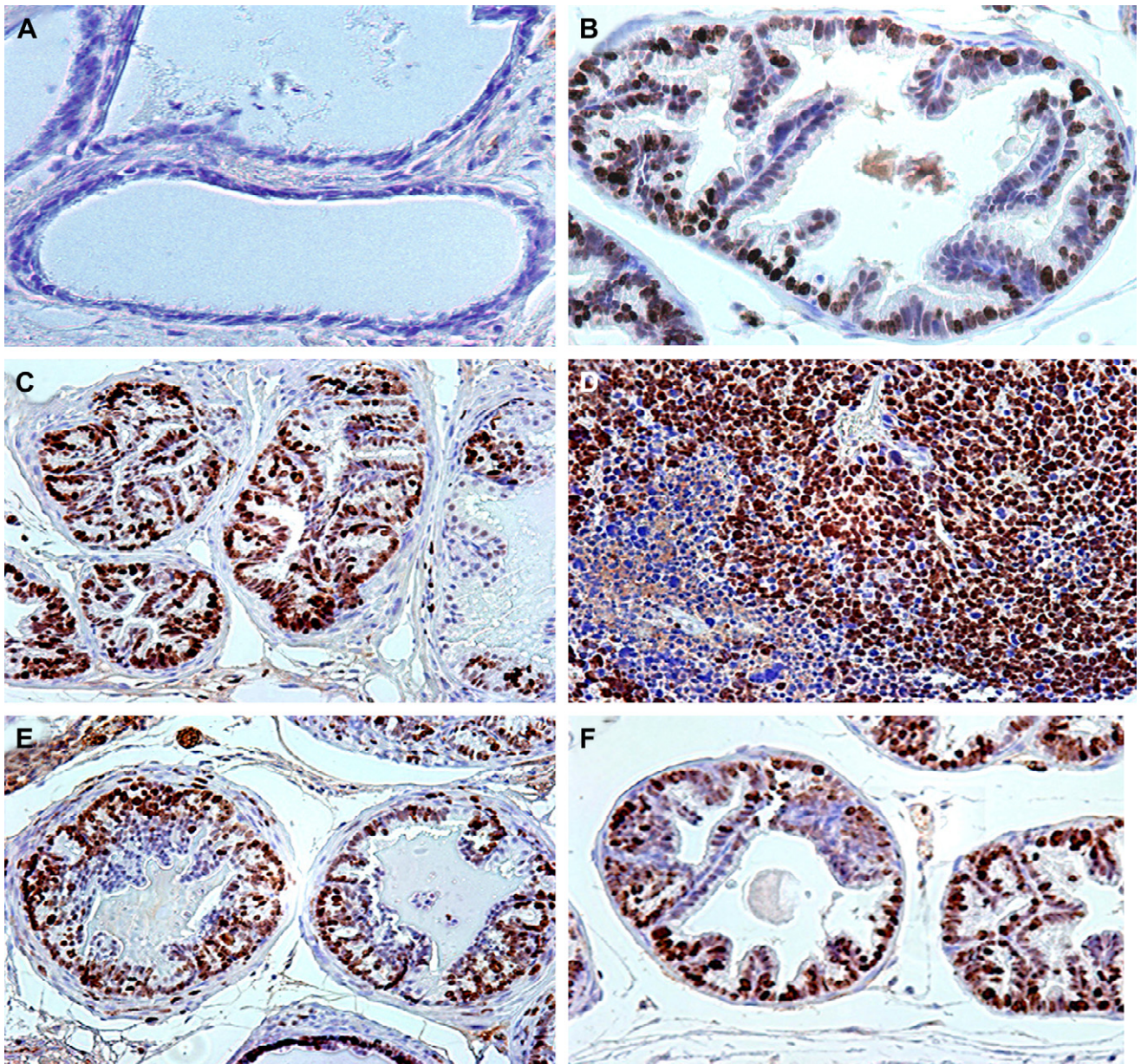
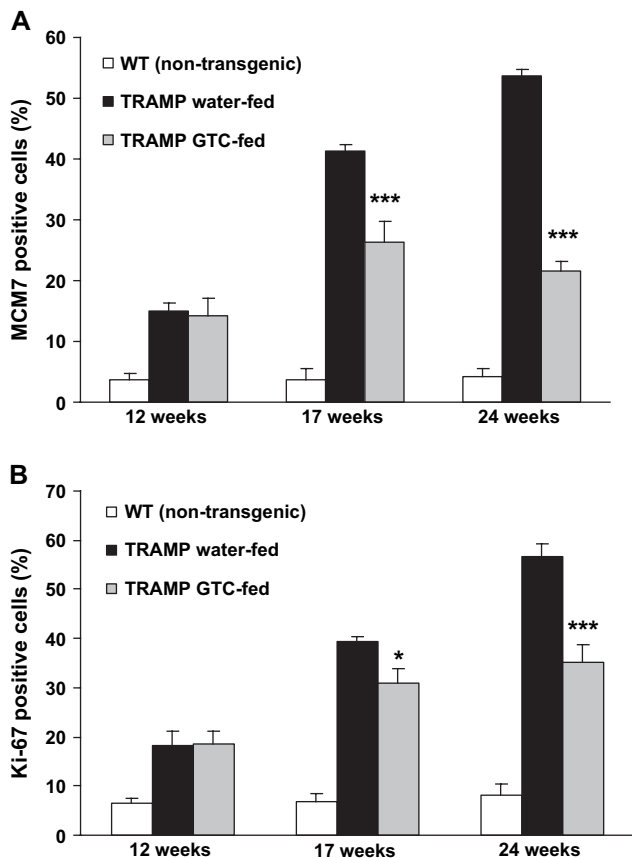


Figure 3 – Immunohistochemical staining for Ki-67 protein in the dorsolateral prostate of WT and TRAMP mice. (A) WT (non transgenic) mouse at 24 weeks of age (20 $\times$ ); (B) TRAMP water-fed mouse at 12 weeks of age (20 $\times$ ); (C) TRAMP water-fed mouse at 17 weeks of age (20 $\times$ ); (D) poorly differentiated prostate cancer in TRAMP water-fed mouse at 24 weeks of age (20 $\times$ ); (E) prostate section of a GTC-fed TRAMP mouse at 17 weeks of age (20 $\times$ ); (F) prostate section of a GTC-fed TRAMP mouse at 24 weeks of age (20 $\times$ ).





**Figure 4 – Percentage of (A) MCM7 and (B) Ki-67 positive cells in the dorsolateral prostates of WT (non-transgenic), TRAMP water-fed and TRAMP treated with GTCs at 12, 17 and 24 weeks. The mean values with standard deviations were used to compare the percentage of positive cells for MCM7 and Ki-67 in each experimental group at the 12, 17 and 24 week time point. Values represent mean  $\pm$  SD of five animals in each group. \* $P < 0.05$ ; \*\*\* $P < 0.001$  compared with aged-matched TRAMP water-fed mice.**

MCM7 protein is upregulated during tumor growth and the chemopreventive efficacy of GTCs in TRAMP mice decreased or delayed expression levels of this protein.

### 3. Discussion

Chemoprevention, by definition, is the means of cancer control in which the occurrence of the disease can be entirely prevented, slowed or reversed by the administration of naturally occurring or synthetic compounds (Brawley, 2002; Klein and Thompson, 2004; Siddiqui et al., 2006). CaP is an ideal disease for chemoprevention studies because it is typically diagnosed in men over the age of 50, and a slight delay in the onset and subsequent progression of the disease could have valuable health benefits (Gupta et al., 2001; Siddiqui et al., 2006). The TRAMP mouse model closely mimics clinical prostate cancer and is considered an ideal model for studying CaP progression (Kaplan-Lefko et al., 2003). Laboratory studies have demonstrated the utility of these mice for CaP chemoprevention and intervention

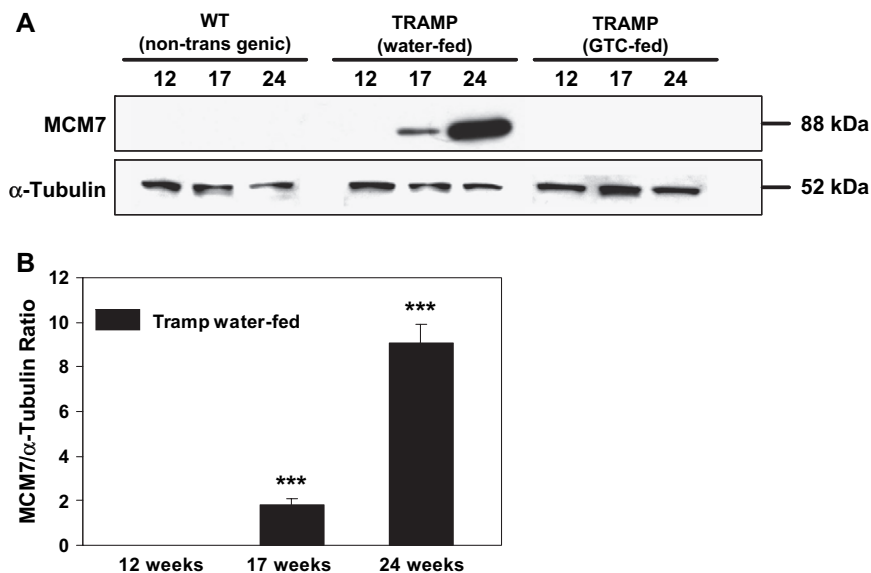
studies (Adhami et al., 2004; Caporali et al., 2004; Saleem et al., 2005).

Using DNA microarray analysis, a group of genes were identified in the TRAMP water-fed mice whose expressions were upregulated relative to WT controls at 17 weeks of age (Figure 1). Among the list of genes, we found MCM7 to be uniquely overexpressed (11.8-fold greater) during the later stages of CaP in the TRAMP water-fed mice as compared to WT control mice. Further analysis of the microarray results suggested that GTC treatment notably inhibited MCM7 expression in the TRAMP water-fed mice during CaP progression.

MCM7 is an essential component of the replication helicase complex (MCM2-7) (Lei, 2005). In a process known as DNA replication licensing, the MCM complex prepares DNA replication by binding to origins of DNA replication during the late M to early G1 phase of the cell cycle (Lei, 2005). Increased expression of MCM proteins has been reported in a variety of malignancies (Gonzalez et al., 2003; Dudderidge et al., 2005; Schrader et al., 2005; Yang et al., 2006), however, only MCM7 has been reported to be highly expressed in prostate cancer (Padmanabhan et al., 2004). Recent prostate tumor studies, found MCM7 overexpression strongly correlated with progression, invasion and relapse (Ren et al., 2006; Honeycutt et al., 2006). In mice, MCM7 overexpression produced primary tumors 12 times larger than controls and resulted in the rapid demise of mice bearing those tumors (Ren et al., 2006).

Our current findings agree with the previous reports. As CaP progressed in TRAMP water-fed mice, MCM7 expression levels continued to increase and at 24 weeks of age, MCM7 expression levels were the highest compared to WT control mice. The majority of TRAMP mice at 24 weeks of age will develop CaP that is invasive and capable of metastatic spread to distant sites such pelvic lymph nodes and lungs (Kaplan-Lefko et al., 2003). The continuous presence of MCM7 throughout the cell cycle not only recruits more cells into the proliferation cycle but may also induce reinitiation of improper DNA synthesis (Ren et al., 2006). Increased proliferation of CaP cells ultimately results in tumor invasion and metastasis leading to significant mortality in humans (Satariano et al., 1998).

One important consideration towards the intervention and prevention of CaP is the development of endpoint surrogate biomarkers that can be correlated with staging of disease along the course of tumor development (Saleem et al., 2005). MCM proteins are expressed at high levels in proliferating cells but not in quiescent somatic cells, which allows for the differentiation of malignant cancer cells from differentiated somatic cells, making these proteins ideal diagnostic markers (Blow and Hodgson, 2002; Lei, 2005). Recently, Padmanabhan et al. (2004) reported MCM7 to be a useful marker of proliferation in CaP, which in our laboratory may be used for evaluating the efficacy of chemopreventive regimens such as GTCs. In our results, MCM7 expression was notably suppressed in the TRAMP mice treated with GTC compared to aged-matched TRAMP water-fed mice. At 24 weeks, our IHC data (Figures 2D–F) indicated that TRAMP water-fed mice had poorly differentiated carcinoma in the prostate, but in the TRAMP GTC-fed mice only PIN lesions were found. Therefore, TRAMP GTC-fed



**Figure 5** – Western blot analysis of MCM7 protein levels in the dorsolateral prostates of 12, 17 and 24-week-old WT (non-transgenic), TRAMP water-fed and TRAMP treated mice with GTCs. Alpha-tubulin antibody was used to show equal loading of the protein samples. Western blot is a representative of one (pooled samples three mice per group) of three experiments performed. (B) Histogram indicates the ratio between MCM7 and  $\alpha$ -tubulin obtained by densitometry analysis of the immunoblot. Data are presented as the mean  $\pm$  SD of three animals in each group. \*\*\* $P$  < 0.001 compared to TRAMP treated with GTCs at 17 and 24 weeks of age.

mice showed a marked delay in CaP tumor development. Western blot analysis, gave the same experimental trend but with different sensitivity (Figure 5).

Reports have suggested that the MCM genes are E2F regulated (Ohtani et al., 1999). Over expression of E2F has been shown to induce cellular DNA synthesis suggesting that genes critically involved in regulation of DNA replication are targets of E2F (DeGregori et al., 1997; Ohtani et al., 1999; Muller et al., 2007), and E2F binding sites have been identified in the human MCM7 promoter (Suzuki et al., 1998). GTCs may effect MCM7 expression because green tea polyphenols have been found to downregulate the protein expression of E2F via retinoblastoma (Rb) pathway (Ahmad et al., 2002). In most human tumors, the Rb pathway is deregulated and when deregulated, Rb activates the E2F family of transcription factors, and thereby inducing the expression of E2F-responsive genes such MCM7 (Ohtani et al., 1999; Ishida et al., 2001; DeGregori et al., 2004; Muller et al., 2007). Green tea treatment in TRAMP mice could be targeting the Rb-E2F pathway, thereby suppressing E2F transcriptional activation, consequently downregulating the expression of genes important for DNA replication such as MCM7. Further studies are needed to address the molecular mechanism and pathways of GTCs on MCM7.

In conclusion, our work and others have implicated MCM7 in prostate cancer progression, growth and invasion. Our present findings indicate that GTC treatment could have a marked effect in delaying the onset of CaP by suppressing the expression of MCM7. In addition, our results support the use of MCM7 expression as a biomarker in testing the efficacy of GTC treatment in CaP chemoprevention. Interactions of GTCs and MCM7 are currently being pursued.

## 4. Experimental procedures

### 4.1. Transgenic animals and GTC chemoprevention

All procedures involving laboratory animals conform to the *Guide for the Care and the Use of Laboratory Animals* published by the US National Institute of Health (NIH publication No. 85-23, revised 1996). Tramp mice, heterozygous for the PB-Tag transgene, were maintained in a C57BL/6 background. The PB transgene is generated using the minimal rat probasin (rPB) regulatory sequence to target SV40 early gene (T and t; Tag) expression specifically to prostatic epithelium (dorsolateral lobes of the prostate) (Kaplan-Lefko et al., 2003). Transgenic males were routinely obtained as [TRAMPxC57BL/6]F1 offspring and characterized as described previously (Kaplan-Lefko et al., 2003). GTCs were extracted from green tea leaves as previously reported, content and purity were assessed by HPLC (EGC 5.5%; EC 12.2%; EGCG 51.9%; ECG 6.1%; total GTCs 75.7%, caffeine <1.0%) (Caporali et al., 2004). GTCs were administered in the drinking water (Caporali et al., 2004) as freshly prepared 0.3% GTC solution every Monday, Wednesday and Friday, starting at the age of 8 weeks. The concentration of GTCs mimics an approximate consumption of 6 cups of green tea per day by an average human (Gupta et al., 2001). Treatment is started at 8 weeks of age because the transgene promoter is initially regulated by the androgens; before then, mice do not display cancer lesions. The animals had access to laboratory chow *ad libitum*.

In this study, three different experimental groups of mice were considered: (i) wild-type (non-transgenic) water-fed (controls); (ii) TRAMP water-fed (cancer is progressing); and

(iii) TRAMP treated with GTCs (cancer is delayed). For each experimental group, 9 animals were sacrificed at 12, 17 and 24 weeks of age to study CaP progression in a time-course fashion. Prostates were quickly removed from each animal; a small portion of the dorsolateral prostate was used for pathological studies and the remaining tissue was homogenized and RNA was extracted for microarray analysis. The dorsolateral prostate tissue used for pathological studies was fixed in 10% zinc-buffered formalin overnight, transferred to 70% ethanol and then embedded in paraffin. Sections of 4  $\mu\text{m}$  were cut, mounted on slides and stained with hematoxylin and eosin for histological analysis. The stained slides were reviewed for the presence of prostate cancer and classified according to previously described grading system (Kaplan-Lefko et al., 2003).

#### 4.2. RNA extraction

Total RNA was extracted from murine prostates using RNA-fast (Molecular Systems, San Diego, CA) according to the manufacturer's instructions and purified on Qiagen columns (Qiagen, Valencia, CA). Three tissue pools were made from each experimental group by pooling three prostate glands together. Five microgram aliquots from each RNA preparation were checked for RNA quality by electrophoresis.

#### 4.3. Microarray analysis

The RNA was processed and hybridized to arrays using standard Affymetrix protocols (Dobbin et al., 2005). The processed RNA was hybridized to GeneChip Mouse Genome 430 2.0 arrays (Affymetrix, Santa Clara, CA). Three independent experimental samples were hybridized for each of the three time points (12, 17, and 24 weeks) for each of the three experimental conditions WT (non-transgenic), TRAMP water-fed, and TRAMP treated with GTCs. All of these arrays were batch analyzed using the robust multi-array analysis (RMA) program in Bioconductor to generate individual signal values for each probeset on each array. Significance analysis of microarrays (SAM) was used to identify genes that appeared to be more highly expressed at 17 weeks than at 12 weeks in the TRAMP water-fed but not WT (non-transgenic) mice. This list was further screened for genes that showed decreased expression at 17 and 24 weeks in TRAMP mice treated with GTCs.

#### 4.4. Immunohistochemical analysis

For immunohistochemistry, five mice were used from each experimental group (WT (non-transgenic), TRAMP water-fed, and TRAMP treated with GTCs) at each time point (12, 17, and 24 weeks of age). Tissues were fixed in 10% zinc-buffered formalin overnight, transferred to 70% ethanol and embedded in paraffin. Four micrometer sections were cut and mounted on slides. Slides were hydrated in xylene, graded alcohol and equilibrated in PBS. Antigen retrieval was performed with sodium citrate (10 mM pH 6) using microwaves for 10 min at 400 W. Endogenous peroxidase activity was quenched with 0.3%  $\text{H}_2\text{O}_2$  in methanol. Non-specific binding was blocked with rabbit normal serum (Pierce,

Rockford, IL, USA). Immunostaining was performed using monoclonal antibodies anti-MCM7 (Santa Cruz Biotechnology, Santa Cruz, CA; dilution 1:50) and anti-Ki-67 clone MIB-1 (DakoCytomation S.p.A, Milan, Italy; dilution 1:25). Slides were washed in PBS and incubated with biotinylated rabbit anti-mouse IgG, F(ab)2 secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, dilution 1:200), followed by peroxidase labeled streptavidin (Amersham Bioscience, Europe GmbH, Milan, Italy). The antigen was visualized by a 10-min incubation with diaminobenzidine tetrahydrochloride (DAB) (Sigma Chemical Co., St. Louis, MO). Negative controls were done by excluding the primary monoclonal antibodies from the reaction. Counterstaining was performed with hematoxylin (Sigma Chemical Co., St. Louis, MO) and cover slips were mounted with Eukitt (O. Kindler GmbH & Co., Freiburg, Germany).

#### 4.5. Assessment of immunostaining

Each slide was evaluated and scored independently by one investigator in a blinded fashion. Counts were performed on high power ( $\times 40$  objective) from the most intensely stained areas. Nuclear staining was considered representative of MCM7 and Ki-67; at least 500 nuclei were counted. The mean values with standard deviations were used to compare the percentage of positive cells for MCM7 and Ki-67 in each experimental group (WT, TRAMP water-fed and TRAMP treated with GTCs) at the 12, 17 and 24 week time point.

#### 4.6. Western blot analysis

Lysates from frozen mouse prostates were homogenized in RIPA buffer (Tris HCl 50 mM, pH 7.4; NaCl 150 mM, Na-deoxycholate 0.25%, NP-40 1%, DNase 50  $\mu\text{g}/\text{ml}$ , RNase 50  $\mu\text{g}/\text{ml}$ ), added with Complete™ protease inhibitor according to the manufacturer's instruction (Roche Diagnostics Corporation, Milan, Italy), and heated at 100 °C for 10 min in SDS-PAGE loading buffer. The equivalent of 50  $\mu\text{g}$  of total protein was loaded on each lane and resolved by electrophoresis on 10% polyacrylamide gel and blotted onto a membrane. Immunoreactive bands were detected with the chemiluminescence blotting substrate (POD) (Roche Diagnostics Corporation, Milan, Italy) using anti-MCM7 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-tubulin (Cell Signaling Technology, Danvers, MA, USA) antibodies.

#### 4.7. Densitometric analysis

Films were scanned on BioRad Molecular Analyst and total volume per band was calculated using BioRad Quantity One 4.2.3 software. Data are presented as mean  $\pm$  SD followed by statistical analysis.

#### 4.8. Statistical analysis

Differences in expression were tested for statistical significance between the experimental groups using a two sample Student's *t* test. *P* values less than 0.05 were considered statistically significant.

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