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Somatic Single Hits Inactivate the X-Linked Tumor Suppressor *FOXP3* in the Prostate

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SUMMARY

Despite clear epidemiological and genetic evidence for X-linked prostate cancer risk, all prostate cancer genes identified are autosomal. Here, we report somatic inactivating mutations and deletion of the X-linked *FOXP3* gene residing at Xp11.23 in human prostate cancer. Lineage-specific ablation of *FoxP3* in the mouse prostate epithelial cells leads to prostate hyperplasia and prostate intraepithelial neoplasia. In both normal and malignant prostate tissues, FOXP3 is both necessary and sufficient to transcriptionally repress c*MYC*, the most commonly overexpressed oncogene in prostate cancer as well as among the aggregates of other cancers. *FOXP3* is an X-linked prostate tumor suppressor in the male. Because the male has only one X chromosome, our data represent a paradigm of "single genetic hit" inactivation-mediated carcinogenesis.

INTRODUCTION

Genetic lesions of several autosomal tumor suppressor genes, including *PTEN* (Sansal and Sellers, 2004; Suzuki et al., 1998), *NKX3.1* (Emmert-Buck et al., 1995; Vocke et al., 1996), and *KLF6* (Bar-Shira et al., 2006; Narla et al., 2005; Narla et al., 2001), have been implicated in the molecular pathogenesis of prostate cancer. In addition, epidemiological studies have suggested a role for X-linked genes that control the susceptibility to prostate cancer (Monroe et al., 1995). Although two loci, one in Xp11.22 (Gudmundsson et al., 2008) and one in Xq27-28 (Xu et al., 1998), have been implicated, the genes in these regions have not been identified. X-linked tumor suppressor genes are of particular interest because the majority of X-linked genes are dose compensated, making a single hit sufficient to inactivate their functions (Spatz et al., 2004). Although we and others have reported X-linked tumor suppressor genes, *WTX1* (Rivera et al., 2007) and *FOXP3* (residing at Xp11.23) (Zuo et al., 2007b) in female cancer patients, none have been identified for cancer in male patients.

In addition to inactivation of tumor suppressors, activation of proto-oncogenes also play a critical role in carcinogenesis. Among them, *c-MYC* (hereby called *MYC*) is known as one of the most commonly overexpressed oncogenes. *MYC* overexpression occurs in more than 30% of all human cancer cases studied (Grandori et al., 2000). However, the mechanism by which *MYC* transcription is increased in the prostate cancer remains unclear. In Burkitt's lymphoma, the *MYC* locus is translocated into a constitutively active Ig locus (Dalla-Favera et al., 1982; Taub et al., 1982), which was found to lead to its transcriptional activation (Erikson et al., 1983). In lung cancer, high levels of gene amplification of the *MYC* locus have been documented (Wong et al., 1986), although such amplification occurred considerably less frequently than overexpression of *MYC* mRNA (Takahashi et al., 1989). Likewise, in breast and prostate cancer, upregulation of *MYC* mRNA was substantially more frequent than amplification of the *MYC* gene (Bieche et al., 1999; Jenkins et al., 1997; Latil et al., 2000). Because *MYC* has been shown to be a target of β-catenin activation (He et al., 1998; Sansom et al., 2007), an appealing hypothesis is that MYC upregulation may be a manifestation of aberrant Wnt

SIGNIFICANCE

The study describes two significant advances. First, we demonstrate *FOXP3* as an X-linked tumor suppressor gene in the male in both human and mice. Because the male has only one X chromosome, our work represents a compelling exception to the widely accepted "two hit" theory for inactivation of tumor suppressor genes. Second, our work demonstrates that FOXP3 is a major transcriptional repressor of *c-MYC* oncogene in the prostate. *FOXP3* inactivation is necessary and sufficient for *c-MYC* overexpression, which is critical for molecular pathogenesis of prostate cancer.

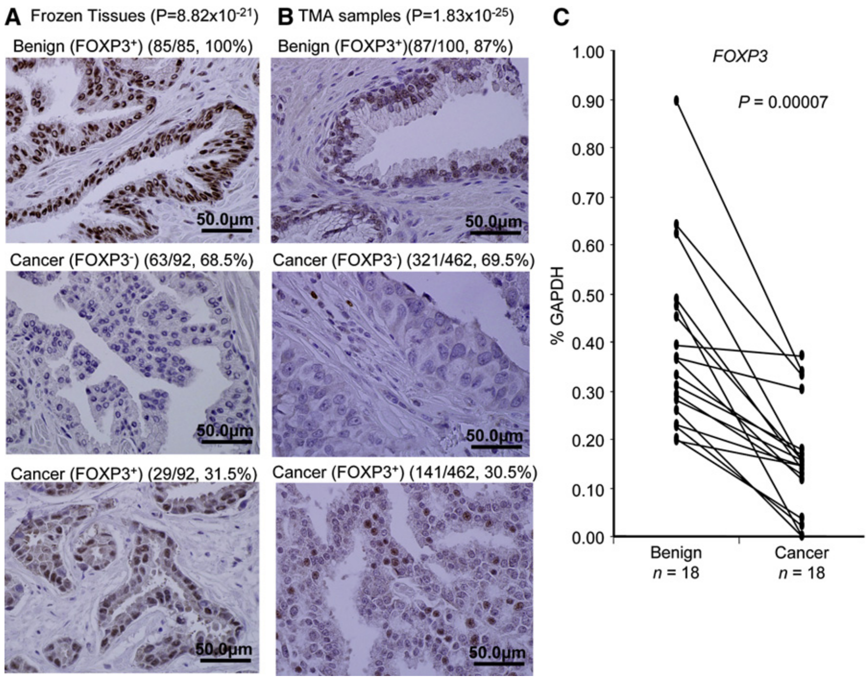

Figure 1. Downregulation of FOXP3 in Prostate Cancer

(A and B) Loss of FOXP3 expression in prostate cancer samples. Data show IHC data for FOXP3 expression in benign versus cancerous tissue in either thawed frozen tissues after short-term formalin fixation (A) or TMA samples after an extended antigen retrieval (B), using monoclonal anti-FOXP3 mAb (236A/E7, 1:100). Statistical significance was analyzed by chi-square tests.

(C) Downregulation of *FOXP3* transcripts in primary cancer tissue in comparison to normal prostate tissue from the same patients (p value by a Wilcoxon two-sample test). Data show real-time PCR quantitation of microdissection samples from 18 cases. The relative amounts are expressed as a percentage of *GAPDH*.

signaling, which occurs frequently in a variety of cancers (Fearon and Dang, 1999). However, a general significance of β-catenin-mediated *MYC* upregulation remains to be demonstrated (Kolligs et al., 1999; Bommer and Fearon, 2007). Therefore, for the majority of cancer types, the genetic change involved in the aberrant *MYC* expression remained to be defined.

MYC overexpression in benign prostate hyperplasia and prostate cancer was documented over 20 years ago (Fleming et al., 1986). Ectopic expression of MYC causes hyperplasia of prostate tissue (Thompson et al., 1989). Further studies demonstrated that *Myc* and *Ras* in combination could induce prostate cancer in mice (Lu et al., 1992). More recently, gene expression profiling of a mouse prostate cancer model induced by *Myc* transgene indicated similarity with human prostate cancer (Ellwood-Yen et al., 2003). Consistent with a role for *MYC* in the pathogenesis of prostate cancer, several whole genome scanning studies have strongly implicated a region 260 kb telomeric to the *MYC* gene in susceptibility to prostate cancer (Amundadottir et al., 2006; Gudmundsson et al., 2007; Haiman et al., 2007a; Haiman et al., 2007b; Witte, 2007; Yeager et al., 2007).

Here, we investigated whether the *FOXP3* gene is frequently inactivated in prostate cancer samples by deletion and somatic mutation. Moreover, we determined the significance of such inactivation by growth inhibition of normal and cancerous prostate cell lines and by identification of FOXP3 targets. The impact of prostate-specific ablation of the *FoxP3* gene in the mouse was also tested.

RESULTS

Somatic Inactivation of the *FOXP3* Locus in Human Prostate Cancer Samples

We first evaluated the expression of FOXP3 in both normal and malignant prostate tissues by immunohistochemistry. Although our previous studies have demonstrated the expression of FoxP3 in the mouse prostate using an affinity-purified anti-FoxP3 peptide antibody (Chen et al., 2008), the FOXP3 expression was not reported in either normal or malignant human prostate tissues by immunohistochemistry (IHC), even though FOXP3 expression on infiltrating regulatory T cells was clearly detectable (Fox et al., 2007; Roncador et al., 2005). As the master regulator of regulatory T cells, *FoxP3* is expressed there at levels comparable to those of housekeeping genes, such as *GAPDH* and *HPRT* (Fontenot et al., 2003; Hori et al., 2003). Because *FoxP3* expression in prostate tissue is approximately 100-fold lower than what was found in regulatory T cells (Chen et al., 2008), we reasoned that the lack of detectable FOXP3 in normal prostate tissue may be caused by low sensitivity of staining and/or tissue processing conditions. Therefore, we first fixed the frozen tissues in 10% formalin for 8–12 hr and screened a large panel of commercially available anti-FOXP3 antibodies for their reactivity to endogenous FOXP3 in epithelial tissues. A shown in Figure S1 (available with this article online), anti-FOXP3 mAb stained prostate epithelial uniformly. However, compared with infiltrating lymphocytes, the level of FOXP3 is considerably lower (Figure S2).

As summarized in Table S1, four commercially available mAbs gave uniform staining of FOXP3 in normal prostate epithelia. The fact that multiple anti-FOXP3 mAbs reacted to FOXP3 demonstrated that FOXP3 is expressed at significant levels in normal prostate tissue. Among them, two (hFOXY and 236A/E7) were also tested and found to react specifically with FOXP3 protein in western blot of lysates made from immortalized mammary epithelial cell line MCF-10A. The specificity of the reactivity to human FOXP3 was further confirmed by comparing reactivity of scrambled and *FOXP3* ShRNA-transduced normal epithelial cell line MCF10A by western blot and by IHC (Figure S3).

Using the uniform fixation and processing conditions, we evaluated the expression of FOXP3 in 85 cases of normal and 92 cases of cancer tissues. As shown in Figure 1A, immunohistochemistry with anti-FOXP3 mAb detected nuclear FOXP3 staining in 100% of the normal prostate tissues tested. In contrast, only 31.5% of the prostate cancer samples show nuclear