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13. ABSTRACT ***(Maximum 200 Words)***

At present, information regarding the contribution of the men-only chromosome, the Y chromosome, to prostate cancer is lacking. The goals of this project are designed to address this question and to identify candidate genes on the Y chromosome involved in prostate cancer. The objectives are: 1) to study the expression of Y chromosome genes in prostate cancer and 2) to evaluate their effects in over-expression in the prostate of transgenic mice. In year 1, we completed the expression survey of all 31 functional genes on this chromosome. Results from this study identified the TSPY gene to potentially play a significant role in prostate cancer development and/or progression. For the next two years of the project, we plan to focus on an expression-function evaluation of TSPY and other Y chromosome genes using transgenic mouse approach, proposed under Objective 2. These studies will provide important information regarding the role of this male-specific chromosome on prostatic oncogenesis and in development of new diagnostic procedures, and/or treatment for this disease. In addition, the generation of transgenic mouse models of prostate cancer will be invaluable in understanding the disease mechanisms, therapeutic strategies and prevention of prostate cancer.

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Table of Contents

INTRODUCTION

The role of the Y chromosome in prostate cancer has been debated on numerous occasions (see reference 1 for review). Both a gain and a loss of this chromosome had been reported in the literature, suggesting the possible existence of an oncogene and tumor suppressor gene respectively. In the past, studies on the function of the Y chromosome in oncogenesis of male-specific organs, such as the prostate gland and testis, are limited due to the lack of information on the genetic content of this chromosome. Recent advances in positional cloning and Human Genome Project have identified most of genes postulated to be on this chromosome, thereby providing the necessary resource for detailed studies on the contribution of Y chromosome genes to male-specific cancers, particularly on prostate cancer (2).

There are two objectives for the present project. First, we evaluate the potential roles of selected Y chromosome genes in prostate cancer by studying their expression patterns on a panel of prostate cancer samples with various degrees of malignancy. Results from these studies, together with information on the properties of the proteins encoded by the Y chromosome genes, should be helpful in determining the most likely candidate genes on this chromosome that may contribute to or be influenced by prostatic oncogenesis. Second, the function(s) of the most significant Y chromosome genes in prostate cancer will be evaluated using a regulated system for transgene expression using transgenic mouse strategies. We hope to correlate the expression-function of these Y chromosome genes to prostate cancer development and progression. These results will be compared to those of known oncogenes, such as the SV40 T antigen and/or c-Myc, expressed in the prostate. These studies should provide significant information on the possible role of the human Y chromosome in prostatic oncogenesis.

BODY

Task 1. To determine the expression/repression and function correlation between the Y chromosome genes and prostate cancer using immunohistochemistry and in situ mRNA hybridization techniques.

The goal of this task is to get a realistic assessment of the potential contribution of the Y chromosome genes in the development and progression of prostate cancer. Both immunohistochemistry and in situ mRNA hybridization techniques have been selected for these studies. Immunohistochemistry is most useful when specific antibodies are available. To this goal, we have generated specific antibodies against the protein products of two human Y chromosome genes, TSPY and SRY, and have obtained another specific antibody against the PHOG gene from Dr. Jay Ellison, Department of Pediatrics, Virginia Commonwealth University. A fourth antibody against a peptide of the mouse Zfy gene had also been generated in our laboratory. However, its cross-reactivity

to the human ZFY protein is limited, therefore we will not use this particular antibody for human ZFY expression study.

In situ mRNA hybridization technique is being used for the remainder of the Y chromosome genes selected for detailed expression studies. This technique does not require the generation of specific antibody, and is based on hybridization of specific probes to mRNA on the tissue sections. To accomplish this goal, we have amplified specific fragments of cDNAs from adult human testis RNA and prostate cancer tissues (2) and subcloned them in plasmid vectors using TA cloning technique. Currently, we have obtained recombinant clones containing the cDNA fragments for the EIF1AY, PRY, CSFR2A, IL3RA, DFFRY, DBY, UTY, TB4Y, SMCY, PRKY, MIC2, IL9R and RPS4Y genes (see 2 for description of genes). Through our previous work, we have also isolated full-length cDNAs for SRY, TSPY, ZFY and those for ANT3, XE7 and PHOG from Dr. Jay Ellison. This collection of Y chromosome gene cDNA probes will be useful in our subsequent expression studies using the in situ hybridization method.

Initially, we proposed to generate antisense and sense riboprobes for in situ mRNA hybridization experiments. However, our recent experience suggests that fresh probes are important for success in this procedure. Therefore, we will generate the respective probes from the above cDNA clones just prior to the actual experimentation. This will insure the integrity and specificity of the respective probes for the in situ mRNA experiments.

Currently, we have performed or initiated experiments on the immunohistochemistry and in situ mRNA hybridization studies on a panel of selected prostate specimens harboring BPH and/or prostate cancer with various Gleason grades. These studies include the TSPY, SRY and the PHOG genes. While these studies are in progress, we have also completed our initial survey of the expression of 31 functional Y chromosome genes on a panel of prostatic samples and cell lines in the presence and absence of hormonal stimulation (2). This study represents the first detailed study on the expression of all 31 functional genes, identified so far, on this male-specific chromosome. The results are significant in refining the course of our efforts for the next two years of this project, discussed later.

The TSPY Gene as a Significant Candidate Proto-Oncogene on the Y Chromosome

As a result of this initial study, one gene on the human Y chromosome has emerged to be the leading candidate that may participate or contribute to the oncogenic process in the prostate. This gene is the testis-specific protein Y encoded (TSPY) gene that was independently isolated in my laboratory several years ago. Since we had generated specific antibody against the recombinant protein of this gene, it was also the first Y chromosome gene that we had studied in details with in situ mRNA hybridization and immunohistochemistry.

Our study demonstrated that TSPY was heterogeneously expressed among the panel of prostatic samples, suggesting that its expression might be influenced by the disease conditions of the respective specimens. Further, TSPY expression was up-regulated by androgen stimulation in the responsive cell line, LNCaP. Using both in situ mRNA hybridization and immunohistochemistry, we demonstrated that TSPY was preferentially expressed in cancerous epithelial cells. The results from these two procedures were in agreement with each other.

TSPY has been mapped on the critical region of the human Y chromosome postulated to harbor a gene(s) that predisposes the dysgenetic gonads of XY sex-reversed females to develop gonadoblastoma. These XY females have lost their functional sex determining gene, SRY, but have retained some Y chromosome materials (including TSPY) in their respective genome (1,3). Among the 5 genes identified within this region for the gonadoblastoma locus on the Y chromosome (GBY), TSPY is the leading candidate since others are either non-functional or have additional copies residing outside this GBY critical region. The GBY gene serves a vital function in the testis, but when it is inappropriately expressed in the germ cells of gonads in a female environment, it predisposes the gonads to develop tumor. Using a specific antibody against the human TSPY protein, we had performed immuno-staining studies on four gonadoblastoma. The results from these studies were exciting and demonstrated a preferential expression of TSPY in tumor germ cells of all gonadoblastoma samples examined (3). We had expanded this study to include two cases of testicular seminoma which also showed a preferential and elevated expression of this gene in tumor germ cells. These findings supported the candidacy of TSPY as the gene for GBY. Further, together with the results of the expression studies on prostate cancer samples (described above) the observations implied that this GBY candidate gene may play an important role in other male-specific cancers, such as seminoma and prostate cancer.

Task 2. To generate animal models for the functional evaluation of Y chromosome genes in prostate cancer

The long-term goal of this task is to establish an efficient system to evaluate the contribution of the most significantY chromosome gene(s), identified under Objective 1, to prostatic oncogenesis. To achieve this Objective, we implement the Tet-Off transgene regulation system in transgenic mice (4) to construct various transgenic mouse models for prostate cancer. In this regulatory system, there are two types of transgenic mice harboring either a transactivator or a responder transgene respectively. Mice harboring either of these genes will be normal. However, when they are crossed with each other to generate bi-transgenic mice harboring both the transactivator and responder transgenes in their genomes, the transactivator gene can stimulate a high level expression of the responder gene. Such a transactivation can be aboished or suppressed by administering a harmless antibiotic, tetracycline or doxycycline, to the animals via their drinking water. Hence, one can compare the physiology and/or oncogenic conditions of genetically identical animals either over-expressing or repressing the responder gene. Any differences between the two groups can be attributed to the over-expression of

the responder transgene. In our case, we will use the candidate Y chromosome gene as a responder gene whose expression is the subject of investigation. We use a prostate-specific promoter, such as that for the rat probasin (PB) (5) or the human PSA gene (6,7), to direct the expression of the transactivator (tTA) gene in the prostate gland, thereby targeting the transactivation of the Y chromosome gene primarily in this organ.

For the past year, we were able to construct the basic transactivator genes directed by the rat probasin promoter, PB-tTA, and the human PSA promoter, PSA-tTA. For the responder gene, we have constructed a basic vector, pTIG, in which the green fluorescent protein (EGFP) can be co-expressed with the responder gene in the same transcriptional unit. We have successfully inserted independently the cDNAs for TSPY, SRY, and SV40 T antigen in this vector, designated as pTIG-TSPY, pTIG-SRY and pTIG-TagΔ respectively.

Next, we tested the ability of these vectors to transactivate a known responder gene, such as TRE-Luc, or to respond to an established transactivator, such as the CMV-tTA, in cell culture system. For PSA-tTA transactivator, we co-transfected it with the TRE-Luc responder gene in LNCaP cells and determined the responses of the luciferase gene in the presence or absence of doxycycline in the culture media. The results showed that PSA-tTA gene was capable of transactivating the luciferase responder gene in the absence of doxycycline. Such response was abolished when doxycycline was included in the media. We were also able to demonstrate that such transactivation was greatly increased when androgen was administered to the cells. Androgen responsiveness of the PSA promoter had been well-documented (6,7). For the responder genes, we evaluated them using a HeLa cell line that had been permanently transfected with a constitutively expressed transactivator (CMV-tTA) gene. When the responder gene, e.g. TIG-TSPY, was transfected transiently to the HeLa Tet-off cells, both the green fluorescent protein and TSPY were transactivated by the endogenous tTA gene to high levels which were repressed in the presence of doxycycline. We detected the GFP and TSPY expression by direct microscopic observation and Western blotting of transfected cells respectively. These experiments established the functionalities of these constructs and paved the way for transgenic mouse construction.

Using the above constructs, we had initiated our transgenic mouse experiments by embryo microinjection in our own laboratory. So far, we had generated a total of 8 transgenic lines harboring the PB-tTA transactivator, 1 line for the PSA-tTA transactivator, and 7 lines for TIG-TSPY responder. Currently, these lines are being maintained and used to establish breeding colonies and homozygous lines. Most recently, we have initiated the construction of transgenic mice for the SV40 T antigen responder gene, TIG-TagΔ. Once established, they will be used to generate bi-transgenic animals harboring a prostate-specific transactivator and the appropriate responder for the proposed studies.

FUTURE DIRECTIONS

The next funding period, we plan to continue the studies proposed in our original proposal. However, recognizing the importance of transgenic mouse models for prostate cancer and the time-consuming procedure on mouse husbandry, we plan to emphasize the studies proposed under Objective 2 for the remainder of the project. For the expression survey of the Y chromosome genes described in Objective 1, we believe we have achieved its goal using a combination of immunohistochemistry and in situ mRNA hybridization (the original methods of choice) and semi-quantitative RT-PCR approach (additional method). We have now analyzed the prostatic expression of all 31 functional genes, so far identified on the human Y chromosome. The most important of these studies identified the TSPY gene to be a potential oncogene in prostate cancer, in addition to it being the candidate gene for GBY. Hence, this important clue must be followed up with detailed molecular, cellular and transgenic mouse studies to elucidate the mechanism(s) by which TSPY may mediate prostatic oncogenesis. This important result from studies conducted under Objective 1 necessitates the immediate emphasis of work proposed under Objective 2.

TSPY encodes a protein with a highly conserved domain homologous to many cyclin B binding proteins (8). This important observation suggests that TSPY may exert its oncogenic properties through modulation of the cell cycle progression in tumorigenic prostatic cells. Such modulation may also be influenced by the hormonal microenvironment of the prostate gland. There are several interesting questions to address. Does over-expression of TSPY in cells stimulate cell proliferation and/or minimizing apoptosis? Does TSPY indeed bind to cyclin B? If so, at what stage of the cell cycle does it exert its effects? Does over-expression of TSPY in the prostate of transgenic mice promote cancer development in this organ? How do its effects compare to those of other known oncogenes, such as the SV40 T antigen, c-Myc or H-Ras? For the remaining two years, we hope to conduct studies proposed in Objective 2 and provide some answers to these important and exciting questions on the role of TSPY and Y chromosome genes in prostatic oncogenesis.

KEY RESEARCH ACCOMPLISHMENTS

- Conduct detailed studies on the expression of all 31 functional Y chromosome genes in prostate cancer and cell lines.
- Identify TSPY to be a putative proto-oncogene residing on the human Y chromosome.
- Establish several transgenic mouse lines harboring either the transactivator or the responder gene, thereby laying the necessary foundation for constructing an efficient system to evaluate the contribution of not only the candidate Y chromosome genes, but also candidates from other human chromosomes, in prostatic oncogenesis.

REPORTABLE OUTCOMES

- First detailed study on the expression of all functional genes, isolated so far, on the human Y chromosome in prostate cancer. This report provides the clues for further studies on the contribution of the Y chromosome to prostate cancer.
  Lau Y-FC and Zhang J (2000). Expression analysis of thirty-one Y chromosome genes in human prostate cancer. Molecular Carcinogenesis 27:308-321.
- Identification of TSPY as a significant candidate gene for the gonadoblastoma gene on the Y chromosome. This study demonstrates the elevated levels of expression of TSPY in gonadoblastoma and testicular seminoma, suggesting a role for this gene on the oncogenesis of respective organs.
  Lau Y-FC, Chou PM, Iezzoni JC, Alonzo JA and Komuves LG (2001). Expression of a candidate gene for the gonadoblastoma locus in gonadoblastoma and testicular seminoma. Cytogenetics and Cell Genetics, in press.
- Demonstration of a preferential and elevated expression of TSPY on prostate cancer. This study establishes the potential universal role of TSPY in male-specific cancers, emphasizing on prostate cancer.
  Lau Y-FC and Komuves LG (2001). Expression pattern of a gonadoblastoma candidate gene suggests a role of the Y chromosome in prostate cancer. In revision.

CONCLUSIONS

Studies conducted in the first year of this project have provided significant information regarding the roles of Y chromosome genes in prostatic oncogenesis. Through a combination of immunohistochemistry, in situ mRNA hybridization and incorporation of the quantitative RT-PCR technique, we completed a preliminary survey on the expression patterns of all 31 functional Y chromosome gene in prostate cancer and cell lines. This survey had identified the TSPY gene to be a strong candidate for a cancer predisposition locus, GBY, on the human Y chromosome and to play a significant role in prostate cancer.

For the next two years, we plan to emphasize on studies under Objective 2 of the proposal and focus on elucidating the mechanisms by which TSPY and other Y chromosome genes may exert their efforts on tumorigenesis of the prostate gland. These studies will also provide important animal models of prostate cancer.

SO WHAT

The studies should be valuable in our fight against prostate cancer. The identification of a Y chromosome gene, TSPY, as a candidate oncogene in prostate cancer may be important for developing new strategies/procedures for diagnosis, pathological grading and clinical prognosis of prostate cancer.

The availability of transgenic mouse models of reversible prostate cancer will greatly facilitate further investigation on the etiology of and the evaluation of therapeutic strategies for prostate cancer in men.

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MOLECULAR CARCINOGENESIS 27:308–321 (2000)

Expression Analysis of Thirty One Y Chromosome Genes in Human Prostate Cancer

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Rapid advances in positional cloning studies have identified most of the genes on the human Y chromosome, thereby providing resources for studying the expression of its genes in prostate cancer. Using a semiquantitative reverse transcription–polymerase chain reaction (RT–PCR) procedure, we had examined the expression of the Y chromosome genes in a panel of prostate samples diagnosed with benign prostatic hyperplasia (BPH), low and/or high grade carcinoma, and the prostatic cell line, LNCaP, stimulated by androgen treatment. Results from this expression analysis of 31 of the 33 genes, isolated so far from the Y chromosome, revealed three types of expression patterns: i) specific expression in other tissues (e.g., *AMELY, BPY1, BPY2, CDY,* and *RBM*); ii) ubiquitous expression among prostate and control testis samples, similar to those of house-keeping genes (e.g., *ANT3, XE7,ASMTL, IL3RA, SYBL1, TRAMP, MIC2, DBY, RPS4Y,* and *SMCY*); iii) differential expression in prostate and testis samples. The last group includes X-Y homologous (e.g., *ZFY, PRKY, DFFRY, TB4Y, EIF1AY,* and *UTY*) and Y-specific genes (e.g., *SRY, TSPY, PRY,* and *XKRY*). Androgen stimulation of the LNCaP cells resulted in up-regulation of *PGPL, CSFR2A, IL3RA, TSPY,* and *IL9R* and down regulation of *SRY, ZFY,* and *DFFRY*. The heterogeneous and differential expression patterns of the Y chromosome genes raise the possibility that some of these genes are either involved in or are affected by the oncogenic processes of the prostate. The up- and down-regulation of several Y chromosome genes by androgen suggest that they may play a role(s) in the hormonally stimulated proliferation of the responsive LNCaP cells. *Mol. Carcinog. 27:308–321, 2000.* © 2000 Wiley-Liss, Inc.

Key words: Y chromosome; gene expression; prostate cancer

INTRODUCTION

The human Y chromosome harbors a small number of genes that play essential and critical roles in the determination, differentiation, and maintenance of male specific organs, such as the testis and prostate gland. Recent positional cloning studies have isolated most of the small number of genes postulated to reside on the human Y chromosome [1–4]. These advances have provided invaluable resources for defining the functions of this chromosome in both normal and diseased physiology of the male-specific organs. Currently, 33 genes have been isolated from this chromosome and they can be divided into three groups (Table 1). The first group consists of X-Y identical genes located on the two pseudoautosomal regions (PARs). PAR1 is located on the telomere of the short arm while PAR2 is situated on the telomere of the long arm. PAR1 and PAR2 contain approximately 2.6 Mb and 400 kb of DNA, respectively. PAR1 harbors 10 genes and PAR2 has two genes. Each PAR gene has an identical gene on the corresponding PARs of the X chromosome. The second group consists of 10 X-Y homologous, but not identical, genes located on the nonrecombining region of the human Y chromosome (NRY). They are single-copy genes and most of which are ubiquitously expressed in many human tissues, including the prostate. Except the amelogenin Y (*AMELY*) gene that codes for an enamel protein, the functions of these genes are still largely unknown. Further, it is still uncertain whether these X and Y homologues are interchangeable in their functionality. The third group has 11 members that are specific to the human Y chromosome. The sex determining region Y (*SRY*) gene has been demonstrated to be the testis determining factor (TDF) that switches on testis differentiation pathway during embryogenesis [5]. Functions of the remaining 10 Y-specific genes have not been defined. Most of them consist of multiple copies on the Y chromosome and express in the testis. They are postulated to serve some functions in male-specific organs, such as regulation of spermatogenesis in the testis [6].

The role of the human Y chromosome in prostate cancer has not been investigated in details. Most studies utilized either classical or molecular cytogenetic techniques but molecular studies on individual genes on this chromosome have been lacking. So far, only the expression patterns of two genes,

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Table 1. Genes on the Human Y Chromosome*

Gene symbol	Gene name	Comments
Group 1: X-Y identical genes		
Pseudoautosomal region 1—short arm		
PGPL	pseudoautosomal GTP-binding protein-like	A conserved GTP-binding protein gene, closest to the telomere of the short arm of sex chromosomes
SHOX/PHOG	short stature homeo-box/pseudoautosomal homeo-box-containing osteogenic gene	A homeo-box containing gene involved in bone growth and stature. Postulated to contribute to Turner phenotype
XE7	XE7	A gene identified from an inactive-X cDNA library, nuclear located product
CSFR2A	GM-CSF receptor 2, alpha subunit	A receptor gene for the granulocyte-macrophage colony stimulating factor, a growth and differentiation factor
IL3RA	Interleukin 3 receptor alpha subunit	A cytokine receptor gene sharing homology to *CSFR2A* gene, IL3 binds to its receptors and promotes hematopoietic cell proliferation
ANT3	adenine nucleotide translocase	A member of the ADP/ATP translocase family, involved in cellular energy metabolism
ASMTL	ASMT-like	A gene encoding a putative fusion protein that shares homology with the bacerial *maf/orfE* at its amino end and to ASMT at its carboxyl terminus
ASMT	acetylserotonin methyltransferase	Coding for an enzyme involved in the last step of melatonin synthesis
TRAMP	TRAMP	A single-exon gene encodes a protein with homology to transposases of the Ac family
MIC2	MIC2	coding for a surface antigen detected by a monoclonal antibody, 12E7. (M, monoclonal; IC, Imperial Cancer Research Fund; 2, order of discovery)
Pseudoautosomal region 2—long arm		
IL9R	Interleukin 9 receptor	Receptor for a growth factor for T cells, erythroid and myeloid precursor cells
SYBL1	synaptobrevin-like 1	Coding for a synaptobrevin-like protein
Group 2: X-Y homologous genes		
Non-recombining Y region—short arm		
RPS4Y	ribosomal protein S4 Y isoform	Ribosomal protein gene postulated to be involved in Turner syndrome; X homologue, *RPS4X*
ZFY	zinc finger Y	Coding for a nuclear transcription factor harboring a DNA-binding domain with 13 zinc fingers, X-homologue, *ZFX*
PRKY	protein kinase Y	PRKY and its X homologue, *PRKX*, are members of cAMP-dependent serine/threonine protein kinase superfamily
AMELY	amelogenin Y	Expressed only in developing tooth buds. X-homologue, *AMELX*. Possibly related to tooth size locus
Non-recombining region—long arm		
DFFRY	Drosophila fat facets related Y	Homologous to *Drosophila* deubiquinating enzyme required for eye development and oogenesis. X homologue, *DFFRX*
DBY	dead box Y	Coding for a protein homologous to RNA helicases harboring conserved DEAD (Asp-Glu-Ala-Asp) motifs
UTY	ubiquitous TPR motif Y	Coding for a protein containing TPR motifs, implicated in protein–protein interaction and as a H-Y antigen
TB4Y	thymosin β4 Y isoform	X homologue sequesters actin
SMCY	selected mouse cDNA Y	Human homologue of the mouse *Smcy* gene coding for a minor histocompatibility H-Y antigen and may serve as a spermatogenic factor
EIF1AY	Translation initiation factor 1A	X homologue is an essential translation initiation factor

Table 1. (*Continued*)

Gene symbol	Gene name	Comments
Group 3: Y-specific genes		
Non-recombining region—short arm		
SRY	sex determining region Y	Evolutionary conserved gene coding for the testis determining factor (TDF)
TSPY	testis-specific protein Y-encoded	Repeated gene coding for a putative cyclin-B binding protein homologous to that of oncogene, *SET*. Postulated to direct the spermatogonial cells to enter meiosis and to be involved in oncogenesisi of the testis tumors and gonadoblastoma.
PRY	PTP-BL related Y	Coding for a protein homologous to PTP-BL, a putative protein tyrosine phosphatase, also repeated on the long arm
TTY1	testis transcript Y1	Repetitive transcripts without any protein coding sequences, also present on the long arm
TTY2	testis transcript Y2	Repetitive transcripts without any protein coding sequences, also present on the long arm
Non-recombining region—long arm		
BPY1	basic protein Y1	Coding for a 125-residue basic protein, repeated gene
BPY2	basic protein Y2	Coding for 106-residue basic protein, repeated gene
CDY	chromodomain Y	Coding for a protein with chromodomain and putative catalytic domain, may modify DNA/protein during spermatogenesis, repeated gene
XKRY	XK related Y	Coding for a protein homologous to XK, a membrane transporter protein, repeated gene
RBM	RNA binding motif	Repeated gene coding for a RNA binding protein, a candidate for the azoospermia factor (AZF)
DAZ	deleted in azoospermia	A gene coding for another RNA binding protein, a candidate for the azzospermia factor (AZF)

*See references 1–4,30 for detailed description of genes on the human Y chromosome and information on additional references.

CSF2RA coding for the α subunit of the GM-CSF receptor and *MIC2* coding for a cell surface antigen, on PAR1 [7], and two Y-specific genes, *SRY* and *ZFY*, had been studied in both prostate adenocarcinoma and benign prostatic hypertrophy (BPH) [8,9]. To initiate a survey of expression patterns of these Y chromosome genes, we have developed an experimental strategy to study the expression of a large number of genes in representative prostate samples and prostatic cell lines. Results from this study identify several Y chromosome genes that are either aberrantly expressed in prostate samples and/or regulated by androgen treatment in the responsive prostatic cell line, LNCaP. This survey hence has identified several candidate genes on the Y chromosome that may either play a role(s) in or be influenced by prostatic oncogenesis.

MATERIALS AND METHODS

Tissues, Cell Lines and RNA Preparation

Tissue biopsies were obtained from the Cooperative Human Tissue Network, Western Division at Cleveland. The prostate samples include one benign prostatic hyperplasia (BPH), two BPHs with cancer foci, and three prostate cancer specimens with various degrees of malignancy (Table 2). Their classifications were based on pathological examination of parallel preparations from the respective samples by attending pathologists of the Institute of Pathology at Cleveland. Since the prostate gland contains various amounts of epithelia and stroma, it is conceivable that normal epithelial cells and stromal elements might be present within the cancerous epithelia and vice versa in these samples. Two prostatic cell lines, LNCaP and PC3, were included in the analysis and they were obtained from the American Type Culture Collection. These cell lines cause tumor formation when they are injected into nude mice. The LNCaP cell line is androgen responsive and can be stimulated by male hormone treatment while PC3 is androgen non-responsive under the same conditions [10,11]. Three testis samples (two normal and one atrophic) were also included as references. Total RNAs were purified from respective tissues and cell lines according to a standard procedure by using the Trizol Reagent (GIBCO-BRL). Purified RNAs were treated with RNase-free RQ1-DNase (Promega Inc.) according to the manufacturer's protocol, phenol-chloroform extracted, precipitated with ethanol, and stored in DEPC-treated water at $-20°C$.

Table 2. Human Tissue Samples

Samples	Diagnosis
Testes	
1. Normal testis	25 year old from autopsy
2. Normal testis	34 year old with atrophic testis and chronic epidymitis
3. Atrophic testis	40 year old with seminoma on left testicle, uninvolved right testicle with atrophy
Prostates	
4. BPH	72 year old, BPH, no patient information
5. BPH + CA	69 year old, adenocarcinoma, Gleason's score 3 + 2 = 5, 25% CA, 50% NEO of remainder
6. BPH + CA	55 year old, Gleason's score, 3 + 2 = 5, 25% CA + 100% BPH on remainder
7. CA	66 year old, % CA unavailable, Gleason's score 3 + 4 = 7
8. CA	61 year old, infiltrating prostate cancer, Gleason's score 3 + 5 = 8, 100% CA in Tumor, 20% NEO of remainder
9. CA	69 year old, prostate cancer, 10% CA of tumor 90% NEO of remainder, other information unknown
Cell lines	
10. LNCaP (androgen responsive)	
11. PC3 (androgen nonresponsive)	
Control	
12. Blank	

CA, cancer; NEO, neoplasm; BPH, benign prostatic hyperplasia.

LNCaP and PC3 cells were cultured in RPMI1640 and Ham F-12 media, respectively, supplemented with 10% fetal bovine serum. They were grown at 37°C in a 5% CO_2 atmosphere. For androgen treatment, LNCaP cells were seeded at 2×10^6 cells per 150 mm culture dish and were cultured for 7 d in RPMI1640 medium supplemented with 10% charcoal-dextran treated (hormone-depleted) fetal bovine serum. The cells were then cultured separately for additional 9 d in the same medium supplemented with a synthetic androgen, R1881, at 0, 10^{-10}, 10^{-9}, or 10^{-8} M concentration. The culture media were changed every 2–3 d. The cells were then harvested for RNA preparation by using the Trizol Reagent (GIBCO-BRL) and RQ1-DNase digestion, as before.

RT–PCR Analysis

PCR primer sets were designed from cDNA sequences of 31 of the 33 genes from the Y chromosome retrieved from the GenBank database [1,2]. Table 3 shows the primer sets for 31 Y chromosome genes and five reference genes. The primers for the repetitive sequences, *TTY1* and *TTY2* were omitted. Their transcripts do not contain any protein-coding capability and were not studied in details here. In the cases of X-Y homologous genes, the respective primer sets were designed to be specific for only the Y-located alleles according to available sequence information. The primers were designed to amplify cDNA fragments of 400–600 bp in size and from the 3′ end of the respective transcripts of the Y chromosome genes. cDNAs were synthesized with 2 μg of total RNAs from the respective samples by using a Superscript II cDNA synthesis kit (GIBCO-BRL). Five percents of the cDNA products were amplified with the respective primer sets in 25 μL of reaction buffer by using the High Fidelity PCR kit (Roche Biochemicals). The touchdown PCR conditions were: 1 × 95°C, 5 min; 2 × (95°C, 30 s; 60°C, 30 s; 72°C, 90 s), 2 × (95°C, 30 s; 58°C, 30 s; 72°C, 90 s), 2 × (95°C, 30 s; 56°C, 30 s; 72°C, 90 s), 2 × (95°C, 30 s; 54°C, 30 s; 72°C, 90 s), 35 × (95°C, 30 s; 52°C, 30 s; 72°C, 90 s), and 72°C, 10 min. The reactions were performed in a multiplexing format on a 96-well microtiter dish by using the PTC-100 Programmable Thermal Control (MJ Research Inc.). Eleven samples and one blank control were arranged along the horizontal row for each Y chromosome gene. For a 96-well format, eight Y chromosome genes were analyzed. The touchdown procedure allowed PCR primer sets of different annealing temperatures to be used in the same time [12–13]. Fifteen microliter of the amplified products were then analyzed by electrophoresis in a 1.2% agarose gel, and visualized by ethidium bromide staining. For the relative expression levels of the Y chromosome genes in LNCaP cells under different hormonal treatments, the last round of amplification was adjusted to 20, 25, 30, or 35 cycles, depending on the relative abundance of the respective Y chromosome gene transcripts in the

Table 3. Primer Sets Used for RT–PCR Analysis

Gene		Primers	Sequences (5′ to 3′)
Y chromosome genes			
Pseudoautosomal regional—X-Y identical genes			
PGPL		PGPL-3	CGAGGTCCACCTTGTTGTGAAC
		PGPL-5	CATCATGGGGTCAGGAGAATCC
SHOX/PHOG		SHOX-3	CGACAAGAGCAAAACTCCATCTG
		SHOX-5	AGGACCACGTAGACAATGACAAGG
XE7		XE7-3	GGAGCATCAGACCATTTTGTACCAD
		XE7-5	CAGCGTACTTGGCACTTCAGTTTC
CSFR2A		CSFR2A-3	ATGTCCATGCCATTCCTACACC
		CSFR2A-5	TTCAACCCTCCCAGCAATGTC
IL3RA		IL3RA-3	ATTACACAGGCATCTCCCATGC
		IL3RA-5	AACCTCCTTCCAGCTACTCAATCC
ANT3		ANT3-F	CACCAAGTCCGACGGCATCCG
		ANT3-G	ACGGTTGAGGATTCTACGTGG
ASMTL		ASMTL-3	TGGGAGGTCAAACACAGTCACC
		ASMTL-5	GATATTGCCAGCAAAGTGGACG
ASMT		ASMT-3	CAATTACCAGAATGCCACCACC
		ASMT-5	AGGAAGGAACCAGTACCTGGAGAC
TRAMP		TRAMP-3	TGAAGAGAACGCTGGCAATGCTAC
		TRAMP-5	GCTTTCAGTCATTCAGGGAAACAC
MIC2		MIC2-3	CTCGGTGGGGTTGACATTTG
		MIC2-5	CACAGGAAAGAAGGGGAAGAGG
IL9R		IL9R-1	TGGAGCCCTTGTCTGAGACTGAAC
		IL9R-2	AGCTCTCAGTTTCCTGGAGCAAGG
SYBL1		SYBL1-3	GAGTTGACTGCTGACCGTATTCG
		SYBL1-5	TGTCGCCGCTAGTCTTATGAGC
Nonrecombining region—X-Y homologous genes			
RPS4Y		RPS4Y-1	AACCTCGGTCGTGTTGGTGTGATC
		RPS4Y-2	GCTGCTACTGCAATTTAGCCACTG
ZFY		ZFY-3	CATCAGCTGAAGCTTGTAGACACACT
		ZFY-5	ATTTGTTCTAAGTCGCCATATTCTCT
PRKY		PRKY-R	AAAACAGACAACATAAAATTACA
		PRKY-F	GACCTTTTCTTCACGTGAC
AMELY		AMELY-6	TGGCTGCACCACCAATCATC
		AMELY-7	GTGAGGCTGTGGCTGAAC
DFFRY		DFFRY-3	GGTGTAAACTGACAAAGATGGGCTC
		DFFRY-5	CCAGCAGCACATCACCTTGAACAAC
DBY		DBY-3	GCATTACTGAGCCAACAGGACATC
		DBY-5	AGGATTTGGTGCCAGAGACTATCG
UTY		UTY-3	GGTATGGTGAAGTTGGTGGTCTTG
		UTY-5	CAGATGCTGTTTCCAGTCCTAACC
TB4Y		TB4Y-3	GCTATTTTCCTTCCCTGCCAGC
		TB4Y-5	GGTGTCGCCTCTTTTTCTGTGG
SMCY		SMCY-3	AAGTACATAAAGAGGGTGGTGGGG
		SMCY-5	GCTTACAACACAAGGATTCAGGCTC
EIF1AY		EIF1AY-3	CGAGGCACAAAGGATGAAAAGG
		EIF1AY-5	GGTTCTACAGTTGGGATTTTGGC
Nonrecombining region—Y-specific genes			
SRY	(Set A)	SRY mid-3	TCTTCGGCAGCATCTTCGCC
		SRY mid-5	TCGCGATCAGAGGCGCAAGA
	(Set B)	SRY-coding3	GGTCTTTGTAGCCAATGTTA
		SRY-coding5	ATGCAATCATATGCTTCTGC
TSPY		TSPY4-3	CCTTGAGAATGTTTATTTTTCATTC
		TSPY4-5	CAGATGTCAGCCCTGATCACTG
PRY		PRY-3	TCCACTCAACGCCTCTCCTTTC
		PRY-5	GGTTATCTTCACAGTGCCTCGGAC
BPY1		BPY1-3	CCTAAACTTAGTTGCTGCTCAGGG
		BPY1-5	CGAAGAAGAAGACTACCAAGGTGG

Table 3. (*Continued*)

Gene	Primers	Sequences (5′ to 3′)
BPY2	BPY2-3	GCATTACCCAGTTTTTGCAGTCAG
	BPY2-5	CCAGATTTTCACAGGTGCTGCTTAC
CDY	CDY-3	GATCGTCAGTGGATTTTGAGCC
	CDY-5	GGTTCCAAACCCCTTATACGACC
XKRY	XKRY-3	GAAGCGGAGCTTAAAGCCAAAG
	XKRY-5	GCATTGCTGATGACATATTCCCTC
RBM	YRRM-13	TACTTTGGTCTTTTCTG
	YRRM-14	ATGATGGCTACGGTGAG
DAZ	DAZ-1	GGAGCAAAGGAGAAATCTGTGGAC
	DAZ-2	TCAAAACCAGCAACTTCCCATG
Reference genes		
Androgen receptor		
AR	AR-3	GTTTCCAATGCTTCACTGGGTG
	AR-5	ACCAATGTCAACTCCAGGATGC
Prostate specific antigen		
PSA	PSA-3	CAGTCCCTCTCCTTACTTCATCCC
	PSA-5	GCACCCCTATCAACTCCCTATTG
SET		
SET	SET-3	AGCTGAGTCCATTATCCCACCCAG
	SET-5	CGCACTTTTGCAGGATGACCTC
Uridine diphosphoglucuronosyltransferase		
UGT	UGT-3	TCTCCAAATGCTATCCTTCCCC
	UGT-5	TCTGGATTGAGTTTGTCATGCG
Hypoxanthine-guanine phosphoribosyl transferase		
HPRT	HPRT-A	CCTGCTGGATTACATTAAAGCACTG
	HPRT-B	GTCAAGGGCATATCCAACAACAAAC

LNCaP cells. The number of cycles was determined empirically for each gene. Such adjustments ensured a semilinear amplification of each Y chromosome gene. The resulting PCR products were analyzed similarly in 1.2% agarose gels. The ΦX174 RF DNA digested with HaeIII was used as molecular weight markers. The PCR primers of several Y chromosome genes, such as *TSPY, RBM,* and *DAZ,* spanned across one or several introns in their respective structural genes. In all cases, PCR amplification of the cDNA samples did not reveal any products specific for the respective genomic DNAs, suggesting that the RQ-DNase treatment was effective in eliminating any contaminant DNA in the original RNA preparations. Hence, all signals obtained from the touchdown RT–PCR analysis were derived from the respective Y chromosome gene transcripts.

Northern Blotting

Twenty microgram of total RNAs from respective LNCaP cells stimulated with the synthetic androgen, R1881, at different dosages were processed for northern hybridization, as described before [25]. cDNA fragments from RT–PCR amplification were subcloned into plasmid vector by using a TA Cloning kit (Invitrogen Inc.) and confirmed by single-passage sequencing with an ABI automated sequencer (DNA Sequencing Core Lab, Howard Hughes Medical Institute). The respective inserts were excised from the plasmids and labeled with a random prime labeling kit (Roche Biochemicals) in the presence of ^{32}P-α-dCTP (Amersham Inc.) and used as probes in the northern hybridization procedure. Hybridization signals were visualized by X-ray autoradiography.

RESULTS

To establish an expression–function correlation, we had developed a strategy for identifying potentially important Y chromosome gene(s) involved in prostate oncogenesis. In this approach, a semiquantitative reverse transcription and touchdown PCR assay [12,13] was used to analyze the expression of Y chromosome genes in a panel of representative prostate and testis samples. cDNAs were initially synthesized from total RNAs of prostate and testis samples and subjected to PCR amplification with respective gene-specific primers in a multiplex format. The touchdown procedure utilized an initial high annealing temperature that was gradually ramped down at decreasing intervals, thereby

allowing primer sets of different T_m to anneal to the correct sequences in the respective samples. For a 96-well microtiter dish, one could examine a total of 11 samples and one blank control (along the horizontal row) for eight gene primer sets (along vertical column). The RNA samples were derived from specimens with different clinical stages or cells under various culture conditions. This strategy, hence, allowed a rapid preliminary evaluation on the expression of a large number of specific genes on a panel of tissue/cell samples.

Expression of Y Chromosome Genes in Normal and Diseased Prostates

Of the 31Y chromosome genes examined, specific PCR products were obtained from the respective gene transcripts (Figure 1A–D). Genes residing on the pseudoautosomal regions were either expressed uniformly (e.g., *PGPL, ASMTL, IL3RA, ANT3, XE7, MIC2,* and *SYBL1*) or minimally (e.g., *SHOX/PHOG, CSFR2A,* and *IL9R*) (Figure 1A). These expression patterns confirmed their ubiquitous and tissue-specific expression, respectively. However, we did observe slightly higher levels of expression of *PGPL* and *CSFR2A* in the prostate cancer samples (#7–9) than in the BPH samples (#4–6). Further, we could not rule out the possibility that these tissue-specific growth factors and receptor genes, i.e., *PHOG, CSFR2A, IL3RA,* and *IL9R,* might be aberrantly expressed in other forms, e.g., metastatic, prostate cancer, or under androgen stimulation, (discussed below). The expression patterns of the X-Y homologous genes were more complex (Figure 1B). The *RPS4Y, DBY,* and *SMCY* genes were expressed relatively uniformly while the remaining six X-Y homologous genes were expressed variably among samples of the testis, prostate, and prostatic cell lines. There seemed to be relatively higher RT–PCR products for *ZFY, PRKY,* and *DFFRY* among the prostate cancer specimens (#7–9) than those among the BPH samples (#4–6) while the reverse was observed for *EIF1AY.* Presumably, these X-Y homologous genes are expressed in most human tissues [2]. Our results demonstrated that their levels of expression could vary between tissues and among samples of the same tissue. Their variability might reflect the physiological states of the respective tissues or samples.

The Y chromosome-specific genes are present only in the male genome and do not have any homologue and/or equivalent genes in the female genome. They are postulated to serve vital functions in male specific organs, such as male sex determination and regulation of spermatogenesis [5,6]. Initially all these genes were demonstrated to be expressed specifically in the testis [2]. Indeed, transcripts from *CDY* and *RBM* genes were primarily detected in the testis (Figure 1C). Both *RBM* and *CDY* had been postulated to serve some functions in spermatogenesis, their testis specific expression was reasonably expected. Apart from their expression in LNCaP cells, the *BPY1* and *BPY2* genes also exhibited a testis-specific pattern. They encode two small basic proteins of unknown functions. The expression levels of the remaining five genes in the testis and prostate samples were heterogeneous (Figure 1C), suggesting that they might represent aberrant expression patterns in these tumor tissues and cell lines. Several aberrant RT–PCR fragments were observed in PC3 cell line. Subsequent cloning and sequencing of these aberrant fragments, however, showed that they were not derived from transcripts of corresponding Y-specific genes. Similar analysis of the gene-specific fragments from other testis and prostate samples confirmed their origins from respective Y chromosome gene transcripts. The lack of Y-specific gene products in PC3 cells might reflect the fact that cytogenetic analysis had failed to reveal an intact Y chromosome in this cell line [11]. The detection of RT–PCR products from both pseudoautosomal and X-Y homologous genes in this cell line (Figure 1B) suggests that perhaps part of this chromosome might have been retained (or translocated to other chromosomes) in its genome. PCR amplification of genomic DNA isolated from PC3 cells indeed showed positive products from selected PAR, e.g., *ANT3, IL3RA,* and *SYBL1,* and X-Y homologous genes, e.g., *ZFY, DFFRY, DBY,* and *AMELY* (data not shown). However, we still cannot rule out the possibility that the RT–PCR products might be derived from transcripts of the respective X alleles or homologues.

Five reference genes, androgen receptor (*AR*) [14], prostate specific antigen (*PSA*) [15], oncogene *SET* [16], uridine diphosphogulucuronosyltransferase (*UGT*) [17] and hypoxanthine phosphoribotransferase (*HPRT*) were analyzed similarly with the touchdown RT–PCR technique by using respective gene-specific primers. The results indicated that both *PSA* and *SET* genes were expressed almost evenly in all prostate samples and the LNCaP cells (Figure 1D). The *AR* expression varied among the samples while that for UGT was predominantly in the LNCaP cell line. In particular, *UGT* gene was down-regulated upon androgen stimulation in this cell line [17]. Most testis and prostate RNA samples seemed to be of good quality, as indicated by their relatively uniform signals for *HPRT* (Figure 1E). The PC3 cells had been demonstrated to lack functional AR and PSA [10,11]. However, recent reports had documented the presence of the respective genes and residual expression of both androgen receptor and PSA molecules in PC3 cells [10,11,43,44]. The detection of both *AR* and *PSA* transcripts with the highly sensitive RT–PCR technique here confirmed the low-level expression of these genes in this androgen-insensitive prostatic cell line.

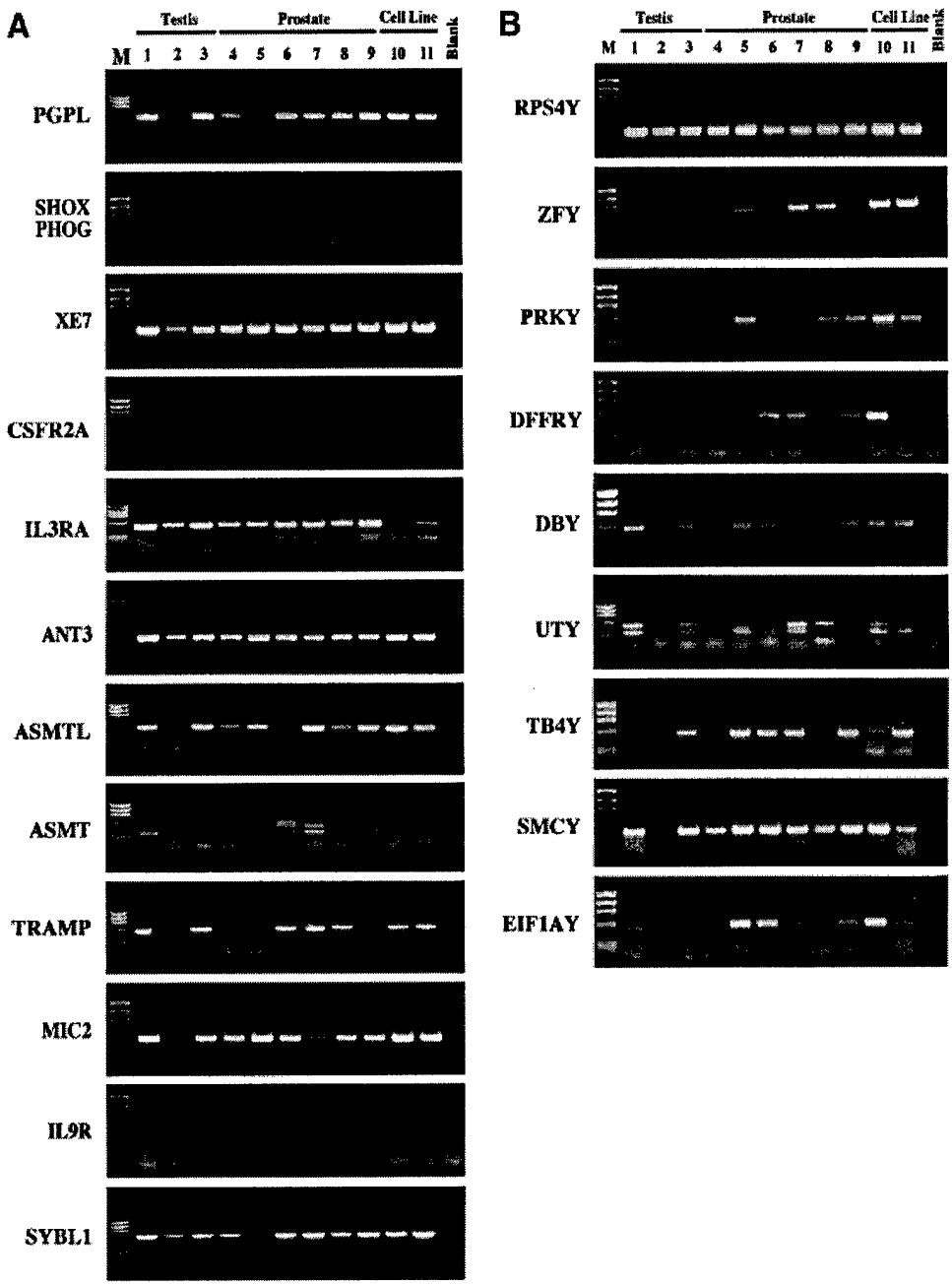

Figure 1. Expression of Y chromosome genes in testis and prostate samples and prostatic cell lines. A. X-Y identical genes on the pseudoautosomal regions. B. X-Y homologous genes on the nonrecombining region of the Y chromosome (NRY). C. Y-specific genes on NRY. D. Reference genes and E. HPRT. The + signs indicate RNA samples treated with reverse transcription, the − signs indicate RNA samples without reverse transcription, before PCR. See Table 2 for samples description and Table 1 and 3 for gene designations and primer information respectively. M = DNA fragments from ΦX174 RF DNA digested with Hae III. The fragments are 1353, 1078, 872, 603, 310, 271–281, 234, 194, 118, 72 bp from top to bottom.

In summary, this initial survey of Y chromosome gene expression in prostate tissues (BPH, BPH + CA, and CA) and prostatic cell lines (androgen responsive and independent) suggested that genes on this chromosome could be divided into several categories. The first category of genes consisted of tissue-specific genes, such as *AMELY*, *SHOX/PHOG*, and *IL9R*. Their expression in prostate tissues was minimal, if detected. Genes for the second category were expressed almost uniformly in most prostate

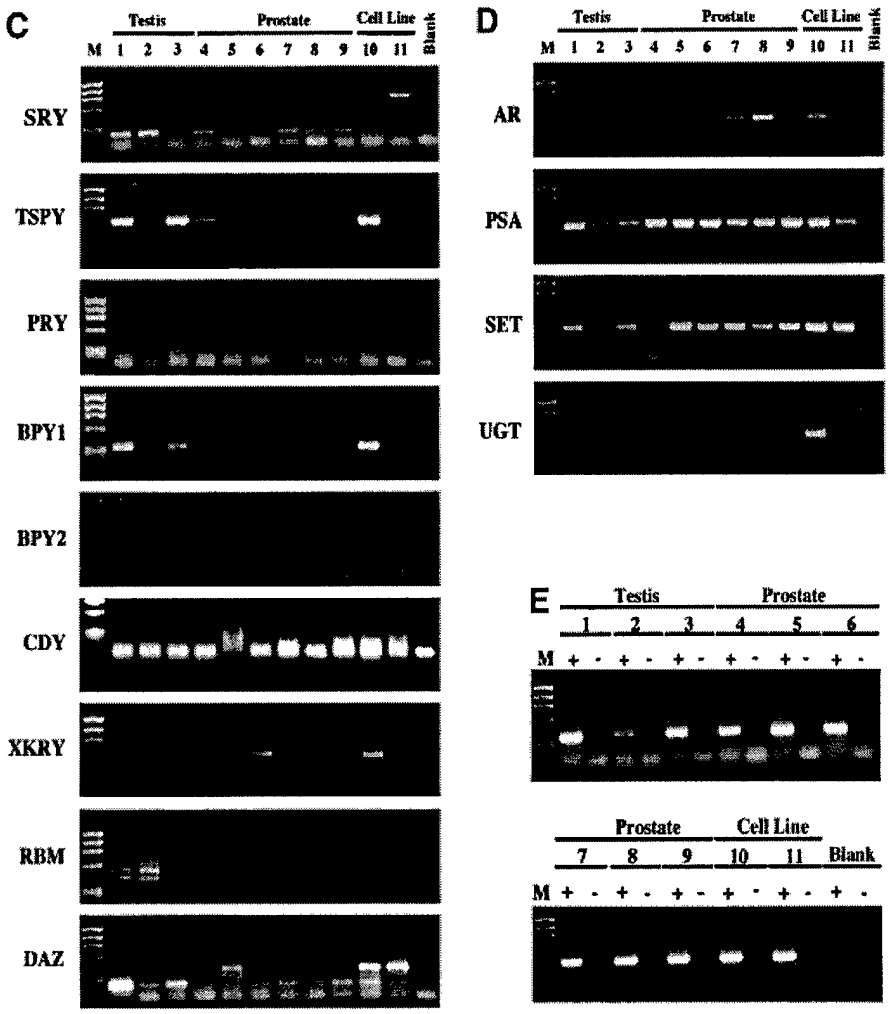

Figure 1. (*Continued*)

tissues (of different pathological classification). They most likely serve essential, and presumably normal, functions in this organ. Members of this category were derived primarily from X-Y identical (PAR), such as *ANT3*, *XE7*, and *MIC2*, and X-Y homologous genes, such as *RPS4Y*, *DBY*, and *SMCY*. The third category of genes consisted of mostly Y-specific and X-Y homologous genes. Their expression levels varied from sample to sample. These genes, particularly the Y-specific ones, are most interesting since their expression patterns may reflect the oncogenic conditions of the prostate samples and cell lines examined.

Androgen Regulation of Y Chromosome Genes

We used the relatively quantitative touchdown RT-PCR technique to evaluate the regulation of Y chromosome genes in the hormonally responsive LNCaP cell line. LNCaP cells were initially cultured in hormone-depleted media. A synthetic androgen, R1881, was then added to the media in different flasks, with concentrations of 10^{-10}, 10^{-9}, and 10^{-8} M. The cells were cultured for an extended period of nine days before they were harvested for total RNA preparation. Numerous studies had demonstrated that similar R1881 treatments stimulate the proliferation of LNCaP cells in a dosage dependent manner [9–11]. The RNA samples from untreated controls and cells treated with R1881 were analyzed with the touchdown RT-PCR procedure by using gene-specific primer sets, as described. Results from this experiment indicated that only a few Y chromosome genes were affected by hormonal treatment of the cells (Figure 2A–D). They include the *IL9R, PGPL, CSFR2A, IL3RA, TSPY, SRY, ZFY, PRY* genes. The steady state transcripts from *TSPY, PGPL, CSFR2A, IL3RA,* and *IL9R* genes increased while those of *SRY, ZFY,* and *PRY*

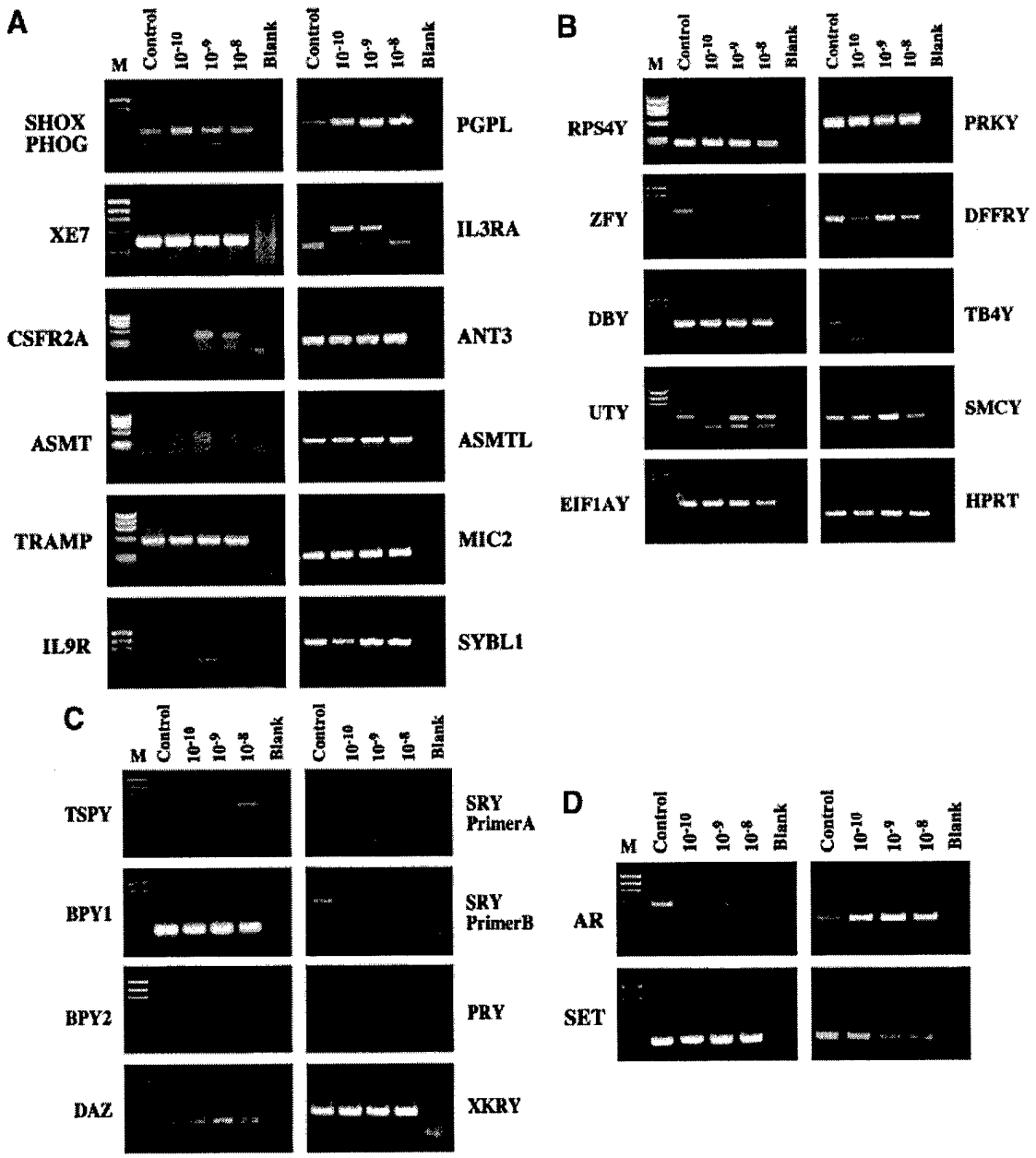

Figure 2. Androgen regulation of Y chromosome gene expression in LNCaP cells, cultured with 0 (control), 10^{-10}, 10^{-9} and 10^{-8} M of synthetic androgen, R1881. A. X-Y identical genes and HPRT control. B. X-Y homologous genes on NRY. C. Y-specific genes on NRY. D. Reference genes. The steady state transcripts from *ZFY*, *SRY*, and *PRY* on the Y chromosome and *AR* and *UGT* reference genes were down regulated while those for *IL9R*, *PGPL*, *CSFR2A*, *IL3RA*, *TSPY*, and *PSA* were up regulated upon androgen treatment of LNCaP cells.

decreased in LNCaP cells by R1881 treatments. The down regulation seemed to be extreme for the *SRY* gene. At a small dose of 10^{-10} M of the androgen, the *SRY* product (Primer A) was totally undetectable while those from the other Y gene transcripts were easily observed. To confirm these results, the PCR amplification was repeated with a second set of *SRY* primers (Primer B). Again, PCR products from androgen treated cells were drastically reduced (Figure 2C). Parallel PCR analysis of other Y chromosome genes, e.g., *XKRY*, *XE7*, *ANT3*, *DBY*, and *RPS4Y*, showed uniform amplification of the respective products.

Transcript levels for the five reference genes, *HPRT*, *AR*, *PSA*, *SET*, and *UGT*, were analyzed similarly in the LNCaP cells. The *PSA* gene showed

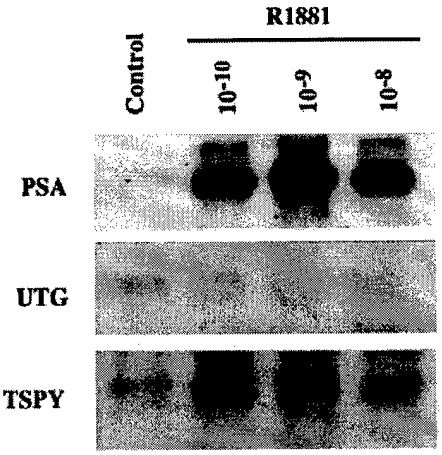

Figure 3. Northern blotting analysis of *PSA*, *UTG*, and *TSPY* transcripts in LNCaP cells treated with the synthetic androgen, R1881. *PSA* and *TSPY* steady state transcripts increased and those for *UGT* decreased under androgen stimulation of LNCaP cells.

a gradual increase while *UGT* and *AR* genes showed gradual decreases of their respective transcripts upon R1881 treatments (Figure 2D). Both *HPRT* and *SET* genes showed no apparent response to androgen regulation. Previously, *UGT* expression had been demonstrated to be down-regulated with androgen treatments in this prostatic cell line [17]. Northern blotting analysis of RNAs from the respective LNCaP cell populations indeed showed increases in steady state transcripts of both *PSA* and *TSPY* genes and a decrease of *UGT* transcripts (Figure 3). Although there was a general correlation between the RT–PCR and northern hybridization results, the relative amounts of transcripts for these genes detected by northern blotting were not as linear as those detected by RT–PCR (e.g., Figure 2). These differences may be attributed to the sensitivity of the respective methods.

DISCUSSION

Prostatic carcinogenesis is a multi-step process mediated by many genetic and environmental components. One or a few of the genetic components could be derived from genes on the Y chromosome. Unfortunately, the assessment on the contribution of this chromosome to the etiology of prostate cancer has been difficult, although the Y chromosome is absolutely essential for male development, including organogenesis of the testis and the prostate gland. There are two main reasons that may be attributed to this problem. First, major portion of this chromosome does not pair with the X chromosome and does not go through any meiotic recombination, thereby making any linkage analysis to be extremely difficult, if not impossible. Consequently, most of the genetic linkage studies on families with hereditary prostate cancer had excluded the Y chromosome in their analyses [48–51]. Second, both classical cytogenetic and/or chromosome painting studies documenting either a gain or a loss of this chromosome in tumor cells derived from prostate cancer [52–55] were relatively crude and did not provide any specific information on the individual Y chromosome genes in the samples analyzed. These results contributed to the controversy regarding the role of this chromosome in prostate cancer. Recently, Tricoli [56] argued that many studies utilizing samples from either short-term cultures and/or tissue sections might give rise to clonal cell selection and nuclear truncation, respectively. He suggested that the Y chromosome was present in most of the prostate cancer specimens. To demonstrate his point, Tricoli [56] had studied the status of the Y chromosome on touch preparations from 42 fresh prostate tumors by using chromosome painting technique. The touch preparation allowed him to transfer intact epithelial tumor cells directly onto a slide without paraffin or frozen sectioning procedures. Results from Tricoli's study demonstrated a gain of the Y chromosome in a single sample and normal number of Y chromosome in the remaining 41 samples, suggesting that loss of the Y chromosome is an infrequent event in prostate cancer. This study, if confirmed independently, supports Tricoli's postulation that this male-specific chromosome is present in the prostate during its oncogenic process. Further, there is genetic evidence suggesting that first-degree males (i.e., father, son, or brother) of a prostate cancer patient who have the same Y chromosome might have a higher probability of getting the disease than those without a first-degree male suffering from prostate cancer [45–47]. These observations, taken together, signify that the genes on this male chromosome might play some roles in prostatic carcinogenesis. Indeed, a cancer locus, termed gonadoblastoma on the Y chromosome or GBY, has long been mapped on the human Y chromosome [57,58]. The GBY locus harbors a gene(s) that predisposes the dysgenetic gonads of XY females to develop gonadoblastoma at a high (>30%) frequency. Significantly, recent isolation of most of the genes residing on the human Y chromosome [1–4] has presented a panel of candidate genes that may play some roles in cell growth, cell cycle regulation, and signal transduction (Table 1). Mutations and/or aberrant expression of one or a few of these genes may contribute to the oncogenic processes not only in gonadoblastoma but also in male-specific organs, including the testis and the prostate gland.

Previous studies had demonstrated the expression of both *SRY* and *ZFY* in over 60% of 30 BPH and prostate cancer samples [7,8]. The present study confirms a similar level (four of six samples) of expression for both genes among the prostatic samples analyzed. In addition, we have further

demonstrated that these two genes, as well as *PRY*, were down-regulated in LNCaP cells upon androgen treatments. *SRY* has been demonstrated to be the gene for *TDF* responsible for switching a male development during embryogenesis [5]. Although the exact mechanism(s) by which *SRY* mediates sex determination is uncertain, a repressor mode of action has been proposed [23]. It's function in adult tissues is still uncertain. If *SRY* serves as a repressor for cell growth or proliferation, its down-regulation would be needed for cellular proliferation events. *ZFY* encodes a transcription factor with DNA-binding zinc fingers [24]. It has a homolog, *ZFX*, on the X chromosome. Both *ZFY* and *ZFX* genes have distinct expression patterns different from each other [25]. The *Zfx* gene in the mouse may play a role in germ cell development since male and female mice harboring a null *Zfx* gene on their X chromosome showed reduced numbers of germ cells in their respective sex organs [26]. *PRY* is a recently isolated repeat gene on the human Y chromosome. It encodes a protein homologous to the protein tyrosine phosphatase, *PTP-BL* [2,27]. Significantly, protein kinases and phosphatases have been postulated to be regulators and signal transducers in cell growth and proliferation [28]. The down-regulation of *PRY* suggests a possible involvement of this putative tyrosine phosphatase in androgen stimulation of LNCaP cells.

Androgen plays an essential role in the development and differentiation of prostate in embryos and prostate cancer pathogenesis in adults. Androgen regulates the expression of genes via the androgen receptor activation pathway [18–22]. Many of these androgen-regulated genes are growth factors and extracellular matrix components that act specifically on the epithelial cells and stimulate their proliferation and differentiation. It has been argued that a similar, if not the same, set of genes may be involved in the regulation of normal development of the embryonic prostate and in oncogenesis of the adult prostate. Although the Y chromosome has been demonstrated to be critical in switching on testis determination and differentiation during embryogenesis [5], its role in prostate development has not been clearly defined. Thus, the identification of androgen-regulated expression of any of the Y chromosome genes would suggest potential candidates important for both prostate organogenesis and oncogenesis.

The present study indeed has identified several PAR genes, *PGPL, CSFR2A, IL3RA, IL9R*, and the Y-specific gene, *TSPY*, to be up-regulated by androgen treatments in LNCaP cells. The *PGPL* gene encodes a putative small GTP-binding protein on the tip of the PAR1 [3]. Numerous studies have demonstrated that small GTPases, such as the Rho family, participate in various cytoskeletal organization and signaling processes and Ras-mediated cell transformation [32–34]. If the *PGPL* product indeed can function as a GTPase, it may potentially play a similar role in cell signaling and transformation. The up-regulation of the three cytokine receptor genes, *IL9R, CSFR2A*, and *IL3RA*, suggests that cytokines and their receptors may play a role(s) in androgen-stimulated proliferation in this prostatic cell line. *IL9R* encodes the receptor for IL-9 [29] while *CSFR2A* and *IL3RA* encode the α subunits of receptors for the granulocyte-macrophage colony-stimulating factor (GM-CSF) and the IL-3, respectively [35–42]. Each α subunit exhibits specific affinity to the respective growth factor but requires heterodimerization with the β subunit to form a fully functional receptor [35–38]. Recent studies have demonstrated that GM-CSF and its receptors are expressed in prostatic cell lines LNCaP, PC3, and DU-145 [39–41]. Further recombinant human GM-CSF stimulates proliferation of the LNCaP cells in vitro [40], suggesting that prostatic cells are responsive to this hematopoietic factor. GM-CSF mediates the proliferative function through its receptor that induces phosphorylation events in the LNCaP cells [40]. Immunostaining experiments localized both α and β subunits of the GM-CSF receptor on the epithelial cells of normal prostate, BPH, and prostatic carcinoma. Both subunits are expressed at low level in normal human prostatic tissue, at substantial level in BPH, and at prominent level in prostatic carcinoma [40]. Hence, GM-CSF and its receptor may play a role in prostatic cell proliferation and oncogenesis. The cytokines, IL3, IL9, and their receptors have been demonstrated to play a role(s) in T cell and other hematologic malignancies [42], their roles in prostatic cells are still unknown.

Using a mRNA differential display technique, Shen and colleagues [7] had recently identified *MIC2* to be a gene up-regulated by androgen treatment of LNCaP cells. *MIC2* expression was responsive to androgen stimulation in the parental LNCaP line, but not in sublines with higher metastatic potential. Immunostaining experiments detected the *MIC2* encoded product, E2, in tissues from patients with primary prostate cancer, but only sporadically in benign prostatic hyperplasia tissues. Our results confirmed that *MIC2* was expressed in mostly prostatic cancer and BPH tissues. However, its steady state RNA level was relatively uniform in LNCaP cells treated with various dosages of R1881. It is uncertain if the differences in our observations and those of Shen and colleagues [7] reflect the methodology and/or cell lines used in these studies.

The *TSPY* gene constitutes the most interesting gene on this chromosome whose aberrant and androgen-responsive expression may be involved in prostatic cell proliferation and oncogenesis in the prostate gland. *TSPY* is a repeat gene with about 20–40 copies on the human Y chromosome [see review, 30]. It is primarily located in the deletion interval 3

on Yp11.2, within a small region where the gonadoblastoma locus has been mapped on this chromosome [57–58]. Hence, *TSPY* is a candidate for *GBY* and whose expression has been demonstrated in gonadoblastoma tissues [30,58, Lau et al., unpublished observations]. TSPY protein shares extensive homology with that of the *SET* oncogene, involved in an intra-chromosomal translocation on chromosome 9 of a patient with undifferentiated acute leukemia [16]. *SET* is a member of a family of cyclin B-binding protein genes that includes the nucleosome assembly protein (*NAP*-1). Some members of this protein family are involved in cell cycle regulation. The *TSPY* gene is expressed normally in spermatogonial cells of the testis and has been hypothesized to direct these germ stem cells to proliferate and/or to enter meiosis [31]. Recently, aberrant expression of *TSPY* has been documented in testicular cancer samples, including germ seminoma and carcinoma-in-situ [30,31]. Our study showed that its expression in LNCaP cells could be stimulated by androgen treatments, suggesting that *TSPY* may be associated with the proliferative activities of these cells under such conditions.

The present survey of Y chromosome gene expression in prostate cancer and BPH samples has highlighted this portion of the human genome that is important for the normal differentiation and/or physiology of male-specific organs. Although there was no clear-cut pattern of Y gene expression specifically related to the degrees of malignancy in our specimens, a small number of genes, such as *TSPY, SRY, PRY, ZFY*, and the cytokine receptors, have been identified to play a potential role(s) in or to be influenced by oncogenesis in the prostate. These results, hence, form the basis for further studies using advanced techniques, such as in situ mRNA hybridization, immunohistochemistry, microdissection and RNA analysis, on clinically defined sets of specimens. Such investigations focusing on individual Y chromosome genes may provide significant insights into the likely contribution of this male-specific chromosome to the initiation and progression of prostate cancer.

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Expression of a candidate gene for the gonadoblastoma locus in gonadoblastoma and testicular seminoma

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Abstract. The gonadoblastoma locus on the Y chromosome (GBY) predisposes the dysgenetic gonads of XY females to develop in situ tumors. It has been mapped to a critical interval on the short arm and adjacent centromeric region on the Y chromosome. Currently

there are five functional genes identified on the GBY critical region, thereby providing likely candidates for this cancer predisposition locus. To evaluate the candidacy of one of these five genes, testis-specific protein Y-encoded (TSPY), as the gene for GBY, expression patterns of TSPY in four gonadoblastoma from three patients were analyzed by immunohistochemistry using a TSPY specific antibody. Results from this study showed that TSPY was preferentially expressed in tumor germ cells of all gonadoblastoma specimens. Additional study on two cases of testicular seminoma demonstrated that TSPY was also abundantly expressed in all stages of these germ cell tumors. The present observations suggest that TSPY may either be involved in the oncogenesis of or be a useful marker for both types of germ cell tumors.

Gonadoblastoma is a rare tumor that arises mostly in the dysgenetic gonads of phenotypic females who harbor some Y chromosome materials in their genome (Page, 1987). The tumor is composed of aggregates of primordial germ cells and sex cord elements resembling immature Sertoli and granulosa cells. These aggregates are surrounded by luteinized ovarian type stroma that may include Leydig or lutein-type cells (Scully, 1953, 1970). Gonadoblastoma has been considered to be an in situ germ cell malignancy from which invasive germ cell tumors can develop (Skakkebaek et al., 1987; Jorgensen et al., 1997; Looijenga and Oosterhuis, 1999; Heerbst et al., 1999).

The prevalence of gonadoblastoma among XY females had led David Page (1987) to hypothesize the presence of a locus, gonadoblastoma locus on the Y chromosome (GBY), that predisposes the dysgenetic gonads of these sex-reversed individuals to develop such in situ tumors. Page further predicted that the gene(s) encoded by the GBY locus has a normal function in the testis and acts as an oncogene only in the dysgenetic gonad. Using a panel of DNAs from XY females with gonadoblastoma, Page had initially mapped the GBY locus to deletion interval 3 on the short arm and intervals 4B-7 on the long arm of the Y chromosome. Additional studies further sublocalized this locus to a small region consisting of ~1–2 Mb of DNA in deletion intervals 3E–3G proximal to and 4B at the centromere and possibly 5E, a proximal interval on the long arm (Salo et al., 1995; Tsuchiya et al., 1995). Among the genes so far isolated from the human Y chromosome (Vogt et al., 1997; Lahn and Page, 1997; Lau and Zhang, 2000), there are five genes residing on this small region: amelogenin Y (AMELY), RNA binding motif Y (RBMY), protein kinase Y (PRKY), protein tyrosine phosphatase PTP-BL related Y (PRY), and testis-specific protein Y-encoded (TSPY). Hence, they are candidates for GBY. AMELY encodes an enamel protein in the tooth buds (Salido et al., 1992). RBMY is a repeated gene with a majority of its functional members residing at interval 6 on the long arm, outside the GBY critical region (Cooke, 1999). It expresses a protein with RNA binding motif in the nuclei of male germ cells. PRKY is a single copy gene coding for a putative cAMP-dependent serine/threonine protein kinase (Schiebel et al., 1997). Both RMBY and PRKY have a homologous gene, RBMX and PRKX respectively, on the X chromosome (Schiebel et al., 1997; Delbridge et al., 1999). PRY is a recently isolated repeated gene family coding for a protein related to the PTP-BL tyrosine phosphatase (Lahn and Page, 1997). Some copies of PRY are present outside the GBY region. TSPY is a repeated gene whose functional members are primarily located in two clusters, TSPYA and TSPYB, within interval 3 (Zhang et al., 1992; Conrad et al., 1996; Vogt et al., 1997) on the short arm and as a single-copy on the proximal region

of the long arm (Ratti et al., 2000). TSPY shares tight homology to a family of cyclin B binding proteins, such as the SET oncoprotein and the nucleosome assembly protein (NAP-1) (Tsuchiya et al., 1995; Schnieders et al., 1996), and has been postulated to play a role in directing the spermatogonial cells to enter meiosis (Schnieders et al., 1996; Vogel et al., 1998). Other cyclin B binding proteins have been demonstrated to be involved in the mitotic process, cell proliferation and/or carcinogenesis (von Lindern et al., 1992; Adachi et al., 1994; Altman and Kellogg, 1997; Carlson et al., 1998; Shin et al., 1999). Hence, aberrant or inappropriate expression of TSPY in dysgenetic gonads may play a role in the etiology of gonadoblastoma.

To evaluate the candidacy of TSPY as the gene(s) for GBY, we have performed detailed expression analysis of TSPY in gonadoblastoma and testicular seminoma, or germ cell tumor, using immunohistochemical techniques. Our results demonstrate that TSPY is preferentially expressed in the germ cells of the tumor aggregates in gonadoblastoma and tumor cells at different stages of testicular seminoma. Its expression pattern is very similar to those of cyclin B1 and another cell proliferative marker, the proliferating cell nuclear antigen (PCNA). These findings, hence, support the postulation that TSPY is a significant candidate for GBY, and suggest a possible role of TSPY in the multi-step carcinogenesis of testicular seminoma.

Materials and methods

Patients

Tissue sections were obtained from archival formalin-fixed and paraffin-embedded tumor specimens. All three gonadoblastoma patients had previously been described (Iezzoni et al., 1997; Hussong et al., 1997). At the time of biopsies, Patient #1 was a 15-year old phenotypic female with a unilateral gonadoblastoma at the left gonad. Patient #2 was a 20-year old phenotypic female with bilateral gonadoblastoma. Both patients #1 and 2 have a 45,X/46, XY mosaic karyotype. Chromosome painting analysis on tissue sections of these tumors showed that most tumor cells harbored a Y chromosome while the stroma showed reduced numbers of cells harboring this chromosome (Iezzoni et al., 1997). Patient #3 was a 15-year old phenotypic female with a 46,XY karyotype. She developed a tumor mass on the left and a streak gonad on the right (Hussong et al., 1997).

Two testicular seminoma specimens were obtained from archival samples at the Anatomic Pathology Section, VA Medical Center, San Francisco. At the time of orchiectomy, Patient #1 was a 49-year old male with a tumor mass confined only to the left testicle. Pathological examination revealed a classical seminoma. Patient #2 was a 49-year old male with an advanced and mixed germ cell tumor composed of seminoma, embryonal carcinoma and yolk sac tumor.

Generation of a specific antibody against TSPY

The entire open reading frame of the human TSPY cDNA (Zhang et al., 1992) was subcloned in-frame in the *Eco*R1 site of the expression vector, pAR(ΔR1) (Blanar and Rutter, 1992), a derivative of the pET3a vector. Recombinant TSPY protein was synthesized in bacterial host, BL21DE3(pLysS) and purified by preparative SDS-PAGE from total lysates of induced bacterial culture. A polyclonal antiserum was generated by repeat immunizations of a New Zealand white rabbit using the service of a commercial vendor (Vancouver Biotechnology, Vancouver, Canada). The specificity of the antiserum was initially assayed by Western blotting against recombinant TSPY protein. The specificity of this antibody to TSPY was further confirmed by both Western blotting and immunocytochemical staining of HeLa cells expressing at high levels a transfected human TSPY gene (Lau, unpublished observations). A polyclonal antibody against the proliferative cell nuclear antigen (PCNA) was purchased from Dako Laboratory, Inc. (Carpinteria, CA). A polyclonal antibody against the human cyclin B1 (synthesized with a bacculuvirus vector in insect cells) was

a gift from Catherine Takizawa and David Morgan, Department of Physiology, UCSF. Both antibodies had previously been demonstrated to be specific for the respective antigens in Western blotting and immunostaining studies (Jin et al., 1998; Takizawa et al., 1999; Kömüves et al., 1999).

Immunohistochemistry of tumor tissue sections

Five micron sections were obtained from archival materials according to established procedure. Immunohistochemical staining was performed as previously described (Kömüves et al., 1999). Heat-induced antigen retrieval pre-treatment was utilized in procedures with PCNA and cyclin B1 antibodies. Immunostaining was conducted in a Tris buffer, pH 7.6, containing 4% of bovine serum albumin, 1% ~~teleostean skin~~ gelatin, 0.1% Tween 20, and 500 mM NaCl. The primary antisera were used at 1:500 to 1:1000 dilution ratios. The binding of the primary antibody was detected by reaction with affinity-purified biotinylated goat anti-rabbit IgG, and visualized by either ABC-peroxidase or ABC-alkaline phosphatase reagents. Commercial substrate kits (Vector Laboratories, Burlingame, CA) were used for the enzymatic detections. For the brown and brick-red signals, the DAB and VECTOR NovaRED kits were used respectively with the ABC-peroxidase reagents. For the red signal, the VECTOR RED kit was used with the ABC-alkaline phosphatase reagents. All signals were dependent on the bindings of the respective primary antibodies and were independent of the substrate kits used. After the immunostaining, the sections were counter-stained with hematoxylin. Omitting the primary antibody in the procedure resulted in no signals. Preabsorption of the TSPY antiserum with excess recombinant TSPY protein abolished the staining. The sections were examined and recorded with a Zeiss Axiophot microscope.

Results

Preferential expression of TSPY in tumor germ cells of gonadoblastoma

Histological analysis of the gonadoblastoma specimens from all three patients showed characteristic aggregates of primordial germ cells and sex chord elements. Immunostaining of tissue sections of all four tumors (two patients with unilateral and one patient with bilateral tumors) showed positive staining of cancerous germ cells within these tumor aggregates of all three patients (e.g. Fig. 1A). In particular, the tumor of patient #3 was organized in smaller aggregates with less sex chord elements (Fig. 1B, C), partially resembling the morphology of some forms of germ cell tumors in the testis (e.g. Fig. 1L, M). The TSPY protein was prominently localized primarily in the cytoplasm of these large cells (Fig. 1C). In all cases, the sex cord elements and the stroma showed very little reactive signals (Fig. 1A, B). Mitotic cells were readily observed on specimens from this patient (Fig. 1D-I). Omission of the primary antibody or pre-absorption of the antiserum with excess recombinant TSPY protein abolished or greatly reduced the reactive staining of the germ cells in these procedures (e.g. Fig. 1I). Analysis of parallel sections with PCNA and cyclin B1 antibodies showed similar staining patterns as those with the TSPY antibody (data not shown). The signals were primarily located on the nuclei for PCNA while those for cyclin B1 seemed to be associated with both nuclei and cytoplasm of the germ cells. The general staining patterns indicated that TSPY, PCNA and cyclin B1 were co-expressed in the same tumor cells.

TSPY expression in the tumor cells at various stages of testicular seminoma

It has been argued that gonadoblastoma is a precursor form of more aggressive germ cell tumors (Skakkebaek et al., 1987; Jorgensen et al., 1997; Looijenga and Oosterhuis, 1999; Herbst et al., 1999). If TSPY is indeed the candidate for GBY, it would potentially participate in the oncogenic process of other germ cell tumors. Previously, Schneiders and

colleagues (1996) had indeed demonstrated an up-regulation of TSPY expression in in-situ carcinoma of the testis, a presumed precursor of germ cell tumors or seminoma. To address the question of whether TSPY may also play a role in more advanced testicular cancer, we have extended our study to include two cases of seminoma using the same immunohistochemical staining technique and specific antibodies against TSPY, PCNA and cyclin B1. They, together, showed various morphological forms potentially representing the different oncogenic stages of these tumors. At the early stages, spermatogenesis might have ceased, thereby depleting normal meiotic cells within the seminiferous tubules. In-situ carcinoma could also occur as a precursor during this initial carcinogenic period (Schnieders et al., 1996). The germ cell-depleted and epithelium-like tubules consisted of a single-layer of cells that might develop into localized multi-layer tumors at various peripheral segments (Fig. 1J, yellow arrows). This transformation progressed until most of the epithelia were lined with multiple layers of tumor germ cells (Fig. 1K). Invasive aggregates of cancerous germ cells eventually evolved from such tubular tumors, abandoning the original tubules (Fig. 1L, blue arrow). In the advanced stages, these aggregates could form a large tumor mass covering a sizable portion of the testis (Fig. 1L, N). Occasionally highly undifferentiated and loosely associated embryonal cells were observed in the late stages of these tumors (Fig. 1O). Presumably, these single tumor cells might have acquired some metastatic properties/potential. Significant expression of the TSPY protein was detected in the tumor germ cells at all stages of these testicular cancers (Fig. 1J-O). Similar to the expression pattern in gonadoblastoma, TSPY was primarily located on the cytoplasm of the tumor cells (Fig. 1M, O). Occasionally, nuclear locations of TSPY were also detected in a few cells. Under each microscopic view, a significant number of mitotic cells could easily be identified (white arrows, Fig. 1M, O). Similar to those in gonadoblastoma, most mitotic cells were stained positively with the TSPY antibody. TSPY expression was at a reduced or insignificant level in the interstitial regions of these testicular cancers.

Immunohistochemical staining of parallel tissue sections with PCNA and cyclin B1 antisera demonstrated similar expression patterns as that of TSPY for both antigens in testicular seminoma (data not shown). Again, PCNA showed a mostly nuclear location while cyclin B1 showed a nuclear and cytoplasmic staining pattern. These observations suggested a possible co-expression of these three proteins in the same tumor germ cells.

Discussion

The mapping of the GBY locus within a small region of the human Y chromosome suggests the existence of a proto-oncogene on this chromosome that predisposes the dysgenetic gonads of XY females to malignancy (Page, 1987; Salo et al., 1995; Tsuchiya et al., 1995). The identification of candidate genes for GBY will not only provide information on the molecular etiology of gonadoblastoma but will also shed light on the contribution of this chromosome to the carcinogenic processes of other male-specific cancers, such as testicular seminoma, germ cell tumors and prostate cancer (Looijenga and Oosterhuis, 1999; Lau, 1999; Lau and Zhang, 2000). Recent studies on TSPY have positioned it to be the most viable candidate for GBY within the critical region on the Y chromosome. First, it is present in the DNAs of gonadoblastoma patients (Salo et al., 1995; Tsuchiya et al.,

1995). Its transcripts had been detected by RT-PCR technique in the corresponding tumor tissues (Tsuchiya et al., 1995) and recently by immunostaining in a single case of gonadoblastoma (Hildenbrand et al., 1999). Second, immunostaining studies had demonstrated its expression in spermatogonia in normal testis (Schnieders et al., 1996), suggesting that TSPY may serve a normal function of directing the spermatogonial cells to enter meiosis, a condition for the GBY gene(s) initially postulated by David Page (1987). Third, the TSPY protein is highly homologous to a family of cyclin B binding proteins, including NAP-1 and SET oncoprotein (Tsuchiya et al., 1995; Schnieders et al., 1996), suggesting that it may bind to this mitotic cyclin and be involved in cell cycle regulation and/or cell proliferation (Shin et al., 1999). Results from the present study demonstrate that TSPY is preferentially expressed in the proliferating germ cells within the tumor aggregates in all four samples from three gonadoblastoma patients, further supporting the candidacy of TSPY for GBY.

Although TSPY protein has previously been detected in some forms of testicular tumors, including in-situ carcinoma (Schnieders et al., 1996), our study on the seminoma specimens has clearly demonstrated the high levels of expression of this GBY candidate gene in all stages of these advanced germ cell tumors. Hence, these results, together with those observed by others (Schnieders et al., 1996), establish a direct relationship between the aberrant TSPY expression and the oncogenic process of testicular cancer. Numerous studies have demonstrated that cyclin B binding proteins, such as SET, are either involved in oncogenesis of acute leukemia, Wilm's tumor or modulation of cell proliferation (von Lindern et al., 1992; Adachi et al., 1994; Carlson et al., 1998; Shin et al., 1999). Although the interactions between TSPY and cyclin B have yet to be demonstrated experimentally, the co-expression of these two molecules on the same tumor cells has raised the possibility that they may indeed interact in vivo. The present study has provided evidence supporting the hypothesis that aberrant expression of TSPY may lead to abnormal cell proliferation and tumor formation (Lau, 1999). Hence, TSPY is not only a key candidate for GBY but may also contribute to the oncogenesis of testicular seminoma .

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Fig. 1. Immunohistochemical localization of TSPY in gonadoblastoma and testicular seminoma. (**A**) TSPY was primarily located in the germ cells of tumor aggregates of all three gonadoblastoma patients. This figure illustrates an example of immunostaining on sections from Patient #1. (**B**) Patient #3 harbored a gonadoblastoma with less organized aggregates. (**C**) An enlargement of boxed area in **B**, showing prominent cytoplasmic locations of the TSPY protein. (**D–I**) Examples of mitotic cells within the gonadoblastoma from patient #3. Cells in **D–G** were stained with TSPY antibody; **H** with PCNA antibody and **I** a control without primary antibody reaction. (**J–O**) Immunostaining of TSPY on tumor sections from seminoma at early (**J**), intermediate (**K**) and late (**L–O**) stages of the testicular cancer. Yellow arrows in **J** point to possible localized tumor growth areas. Blue arrow in **L** indicates a tubule being abandoned by the invasive growth of the tumor (boxed area enlarged in **M**). **N** Shows an advanced tumor area consisting of large mass of tumor cells and highly undifferentiated and loosely associated embryonal cells (boxed area enlarged in **O**). White arrows in **M** and **O** point to mitotic tumor cells. Positive signals are brick red in **A**; chocolate in **B** and **C**; brown in **D–H**; and red in **J–O**. Bars indicate 50 μm in **A, C, M** and **O**; 200 μm in **B, J, L,** and **N**; and 10 μm in **D–I**.

Note added in proof. Recently Stuppia et al. (2000) have demonstrated by cloning and sequence analysis that PRY gene spans 25 kb in size and contains 5 exons. The functional copies of PRY are located in interval 6 on Yq while those on Yp retain only exon 1 and 2 and are likely non-functional.

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