

UCSF

UC San Francisco Previously Published Works

Title

The potential contributions of a Y-located protooncogene and its X homologue in sexual dimorphisms in hepatocellular carcinoma

Permalink

<https://escholarship.org/uc/item/0sk7m210>

Journal

Human Pathology, 45(9)

ISSN

0046-8177

Authors

Kido, Tatsuo
Lo, Regina Cheuk-lam
Li, Yunmin
[et al.](#)

Publication Date

2014-09-01

DOI

10.1016/j.humpath.2014.05.002

Peer reviewed



Original contribution

The potential contributions of a Y-located protooncogene and its X homologue in sexual dimorphisms in hepatocellular carcinoma[☆]



Tatsuo Kido PhD^{a,b}, Regina Cheuk-lam Lo MD^c, Yunmin Li PhD^{a,b}, Joyce Lee BS^c,
Z. Laura Tabatabai MD^d, Irene Oi-lin Ng MD^c, Yun-Fai Chris Lau PhD^{a,b,*}

^aDepartment of Medicine, Veterans Affairs Medical Center, San Francisco, CA, 94121, USA

^bInstitute for Human Genetics, University of California, San Francisco, San Francisco, CA, 94121, USA

^cDepartment of Pathology and State Key Laboratory for Liver Research, The University of Hong Kong, Pokfulam Rd, Hong Kong, China

^dDepartment of Pathology, Veterans Affairs Medical Center, San Francisco, CA, 94121, USA

Received 25 February 2014; revised 8 May 2014; accepted 9 May 2014

Keywords:

Sex chromosomes;
Gonadoblastoma gene;
TSPY;
TSPX;
Hepatocellular carcinoma;
Sexual dimorphism

Summary There is a significant sex disparity favoring males among hepatocellular carcinoma (HCC) patients. Although various risk factors have been identified, the exact etiology of such sexual dimorphism(s) in HCC is uncertain. Previous studies showed that overexpression of the Y-located protooncogene, *testis-specific protein Y encoded (TSPY)*, promotes cell proliferation and oncogenesis whereas its X-located homologue, *TSPY homologue X (TSPX)*, retards cell cycle and oncogenic progression. Furthermore, TSPX promotes proteasomal degradation of hepatitis B virus–encoded X oncoprotein and hence could serve as a tumor suppressor in virus–associated HCC. Using immunohistochemistry and reverse-transcription polymerase chain reaction analysis, we had examined the expression of TSPY and TSPX with reference to other established biomarkers in HCC and related liver cancers. Our results demonstrated that 55 (19.2%) of 287 male cases were TSPY positive in immunohistochemistry of tissue arrays, and 15 (46.9%) of 32 male cases were TSPY positive in reverse-transcription polymerase chain reaction analysis of clinical samples. TSPY expression was closely associated with the expression of HCC biomarkers, such as glypican 3. In contrast, TSPX expression was down-regulated in 54.5% of total tumor/nontumorous paired samples (18/33) and negatively associated with those of TSPY, glypican 3, and forkhead box M1 (FOXO1) and was positively associated with that of a tumor suppressor, insulin-like growth factor binding protein 3. The present findings support the hypothesis that the oncogenic events leading to an ectopic activation of the Y-located protooncogene TSPY and/or inactivating mutation/epigenetic silencing of the X-located tumor suppressor gene TSPX could collectively contribute to the sexual dimorphism(s) in HCC and related liver cancers in male-biased manners.

© 2014 Elsevier Inc. All rights reserved.

[☆] This work was partially supported by a grant (R21CA52589) from the National Cancer Institute (Bethesda, MD, USA) and a Merit grant from the Department of Veterans Affairs (Washington DC, USA) to Y. F. C. L. who is a research career scientist of the Department of Veterans Affairs.

* Corresponding author: Laboratory of Cell and Developmental Genetics, Department of Medicine, Veterans Affairs Medical Center—111C5, University of California, San Francisco, 4150 Clement St, San Francisco, CA 94121-1545.

E-mail address: chris.lau@ucsf.edu (Y. F. C. Lau).

1. Introduction

Sex disparities in incidence, progression, and treatment responses are frequently observed in numerous types of cancers [1]. Although sex hormones and differential parental imprinting could play significant roles in the pathogenesis of these sex-biased cancers, the etiologies of sexual dimorphisms in human oncogenesis are still largely unknown. Recent studies suggest that sexual dimorphisms in human cancers could have genetic and/or epigenetic components in their respective oncogenic processes [2]. Significantly, mutations and/or epigenetic dysregulation of genes on the sex chromosomes could contribute to the complex etiologies of nonsex-specific cancers. Because man possesses a single X chromosome, any mutations or abnormal silencing of an X-located tumor suppressor would result in severe deficiency in tumor suppression function of the inactivated gene [3]. Conversely, inappropriate activation of an oncogene or tumor-promoting gene on the male-only Y chromosome could exacerbate the tumorigenic process(es) [4,5]. One prime example of such sexually dimorphic cancers is the hepatocellular carcinoma (HCC), in which there are significantly high male/female ratios among the patient populations worldwide [6]. Hepatocarcinogenesis could be lengthy processes and involve numerous risk factors, including chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infections and oncogenic actions, genetic predisposition, and environment-gene interactions [7,8]. Currently, the exact etiologies of HCC are still uncertain, despite the identification of various viral, environmental, and genetic risk factors.

The *testis-specific protein Y-encoded (TSPY)* gene is a repeated gene located at the critical region harboring the gonadoblastoma locus on the Y chromosome [5,9]. It is postulated to serve a normal function(s) in spermatogonial cell renewal and meiotic division [10]. However, when it is ectopically expressed in germinal cells in an incompatible niche, such as a dysfunctional testis or ovary, it could exert proliferative properties on the affected cells and contribute to the overall oncogenic processes of gonadoblastoma and other germ cell tumors. Indeed, *TSPY* expression has been documented in numerous types of germ cell tumors, including gonadoblastoma, seminoma, and carcinoma in situ, the precursors for all testicular germ cell tumors as well as extragonadal intracranial germ cell tumors of male patients [11–13]. Ectopic expression of a *TSPY* transgene in the ovary of transgenic mice results in gonadoblastoma-like structures in the ovarian environment [5]. Its expression in other nongonadal cancers, such as HCC and melanoma, has been reported [14,15], suggesting its potential role in the oncogenesis of these types of human cancers in a male-biased manner. Significantly, there is a single-copy homologous gene on the X chromosome, termed *testis-specific protein Y encoded-like 2 (TSPYL2)*, *cell division autoantigen 1 (CDA1)*, *differentially expressed nucleolar transforming growth factor β 1 target protein (DENTT)*, or

TSPY *homologue X* (hereon referred as *TSPX*), which possesses contrasting properties as those of *TSPY* [5,16,17]. *TSPX* expression is repressed in lung cancer, as compared with normal tissues, whereas overexpression of *TSPX* retards cell growth and proliferation, reduces colony formation and migratory potential, and stabilizes and activates the tumor suppressor, p53 [18,19]. Importantly, *TSPX* interacts with the HBV-encoded X oncoprotein (HBx), and promotes its proteasomal degradation [20]. Hence, *TSPY* and *TSPX* could serve opposing functions in hepatocarcinogenesis.

To further establish the potential roles of *TSPY* and its X homologue, *TSPX*, in HCC, we have examined the *TSPY* and *TSPX* expression with references to other established biomarkers in HCC and related liver cancers, using immunostaining on tissue microarray and individual tissue sections, conventional and quantitative reverse-transcription polymerase chain reaction (qRT-PCR) on RNAs derived from HCC samples and corresponding nontumorous liver tissues, and complementary DNA (cDNA) sequencing analysis. Our results show that *TSPY* could be aberrantly expressed in tumor regions of HCC but not the corresponding adjacent nontumorous tissues and positively correlated with expression of an HCC cancer cell biomarker, *glypican 3 (GPC3)* [21] and an oncogenic transcription factor, *FOXM1* [22,23]. *TSPX* expression was negatively correlated with those of *TSPY*, *GPC3*, and *FOXM1* but positively correlated with *insulin-like growth factor binding protein 3 (IGFBP3)*, a known tumor suppressor for HCC [24,25]. Our data suggest that ectopic *TSPY* activation and/or *TSPX* mutation/inactivation could collectively contribute to the complex mechanisms of sexual dimorphisms in the initiation and progression of HCC.

2. Materials and methods

2.1. Tissues and sera

Tissue microarray slides were purchased from US Biomax (Rockville, MD), which provides deidentified information on the pathology, Edmondson grading, and tumor stage of each specimen and the sex and age of the patient. A total of 433 specimens, consisting of 358 cases of HCC (287 males and 71 females), 43 cases of cholangiocarcinomas (24 males and 19 females), 12 cases of other hepatocarcinomas (9 males and 3 females), including 6 mixed carcinomas, 2 each of papillary cystadenocarcinomas and adenosquamous carcinomas, and 1 each of squamous cell carcinoma and sacromatoid carcinoma), and 20 normal liver samples (13 males and 7 females) (Table 1) were spotted in 1-mm diameter circular duplicates on the tissue microarray. These tissue microarrays were initially used for detection of *TSPY* expression with immunohistochemistry using a *TSPY*-specific monoclonal antibody [26,27].

Table 1 Immunohistochemical analysis of TSPY on tissue microarray of HCC and related tumors and normal liver specimens

TMA samples		HCC	Cholangiocarcinoma	Other hepatocarcinomas ^a	Normal liver
Total cases (male + female)		358	43	12	20
Male	Total	287	24	9	13
	TSPY positive	55 (19.2%)	5 (20.8%)	0 (0%)	0 (0%)
Female	Total	71	19	3	7
	TSPY positive	0 (0%)	0 (0%)	0 (0%)	0 (0%)

Abbreviation: TMA, tissue microarray.

^a Six mixed carcinomas, 2 each of papillary cystadenocarcinomas and adenosquamous carcinomas, and 1 each of squamous cell carcinoma and sacromatoid carcinoma.

Paired HCC and corresponding nontumorous tissues were obtained from patients who underwent surgical resection between 1992 and 2001 at Queen Mary Hospital, Hong Kong [28]. Thirty-two cases were men, and 7 cases were women with a mean age of 55.3 and 58.3 years, respectively (ranging from 29 to 82 years) (Table 2). Tissue specimens were fixed in 10% neutral formalin and then dehydrated with ethanol series. The fixed tissues were embedded in paraffin, and 5- μ m sections were prepared according to standard procedure. Some were stained with hematoxylin and eosin for pathologic evaluation and Edmondson grading according to standard criteria. The preoperative serum alpha-fetoprotein (AFP) level was assayed by the Department of Pathology at Queen Mary Hospital. The studies were performed under protocols, approved by Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster and the Institutional Committee on Human Research, Veterans Affairs Medical Center and the University of California, San Francisco.

2.2. Reverse-transcription polymerase chain reaction, qRT-PCR, and DNA sequencing analysis

Tissue samples were snap frozen in liquid nitrogen immediately after resection and kept at -80°C . The nontumorous liver samples were taken distal to the HCCs as described previously [28]. Total RNA was extracted from human HCCs and adjacent nontumorous liver samples using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA samples were isolated from 39 HCC samples and 36 adjacent nontumorous liver samples (29 male cases and 7 female cases). After RQ1-DNase treatment (Promega, Madison, WI) to remove any contaminant DNA, cDNA was synthesized from 1 μ g of total RNA using the Transcriptor Reverse Transcriptase kit with oligo(dT)₁₅ primer (Roche Applied Science, Indianapolis, IN). The synthesized cDNA samples were individually analyzed with polymerase chain reaction (PCR) and gene-specific primers using a touchdown procedure [26,29]. The

Table 2 Correlation between TSPY expression and clinical parameters

	Male		Female	
	TSPY positive	Total	TSPY positive	Total
	(15 cases)	(32 cases)	(0 case)	(7 cases)
Age	56.1 $\pm$ 8.8 (SD)	55.3 $\pm$ 10.1 (SD)	—	58.3 $\pm$ 17.7 (SD)
Edmondson grading ^a				
I/II	7 (47%) ^c	15	0	4
II/III	7 (44%) ^c	16	0	3
Serum AFP ^a				
<200 μ g/L	7 (39%) ^c	18	0	5
$\geq$ 200 μ g/L	8 (57%) ^c	14	0	2
<2000 μ g/L	9 (38%) ^c	24	0	6
$\geq$ 2000 μ g/L	6 (75%) ^c	8	0	1
HBV transcript ^b				
HBV RNA negative	2 (29%) ^c	7	0	3
HBV RNA positive	13 (52%) ^c	25	0	4

^a One TSPY-positive case was not graded or measured.^b HBV RNA expression was analyzed by RT-PCR.^c Percent of total cases in the respective category.

reverse-transcription polymerase chain reaction (RT-PCR) products were analyzed by electrophoresis on agarose gels. Quantitative real-time PCR was performed using the GoTaq qPCR Master Mix (Promega) and MyiQ real-time PCR detection system (Bio-Rad, Hercules, CA). The primer sequences were *TSPY* primers (analysis of splice variants): 5'-AAGCTTATGCGCCCTGAGGGCTCGCT-3' and 5'-AGC CCTGTATTCTGTGATGT-3'; *TSPY* primers (qRT-PCR analysis): 5'-ATGAAGTGGAGGCCTATCGC-3' and 5'-CCTTGAGAATGTTTATTTTTCATTCC-3'; *HBV* (region: 219-704 nucleotides) primers, 5'-TGACAAGAATCCTCA CAATACCGC-3' and 5'-CGAACCCTGAACAAATGG CAC-3'; *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) primers, 5'-CCACCCATGGCAAATTCATGGC A-3' and 5'-TCTAGACGGCAGGTCAGGTCCACC-3'; *cyclin B1* (*CCNB1*) primers, 5'-CTGTGTCAGGC TTTCTCTGA-3' and 5'-GCATCTTCTTGGGCACACAA-3'; *GPC3* primers, 5'-GTTATGTGCCATTCTCAACAA CG-3' and 5'-CAGTCTCCACTTTCAAACCCCTTCC-3'; *FOXMI* primers, 5'-GCTTGCCAGAGTCCTTTTGC-3' and 5'-CTGAGTTCTCGTCAATGCCAGTC-3'; and *IGFBP3* primers, 5'-AACTTCTCCTCCGAGTCCAAGC-3' and 5'-GCCCATACTTATCCACACACCAGC-3'.

All gene expression levels were compared with the corresponding levels of *GAPDH* in the same specimens, and differential expression levels between tumor and nontumorous sample pairs was then calculated and further analyzed with the Student *t* test. Any differential expression level with a $P \leq .01$ was considered to be statistically significant.

For the sequencing analysis, the RT-PCR products were extracted from gel after electrophoresis using the Wizard SV Gel and PCR Clean-Up System (Promega) and ligated with a pGEM-Teasy cloning vector (Promega) for DNA sequencing in both directions with an automatic sequencer. The sequences were analyzed with the MacVector software (version 12.7; MacVector, Cary, NC).

2.3. Immunohistochemistry and immunofluorescence

Immunohistochemistry and immunofluorescence were performed as described previously [12,13,30], using a mouse anti-TSPY monoclonal antibody (1:2000), a mouse anti-GPC3 monoclonal antibody (1:100; BioMosaics, Burlington, VT), or anti-Ki-67 rabbit polyclonal antibody (1:100; Thermo-Fisher Scientific, Waltham, MA). The immunoreactive signals were visualized either by the SuperPicTure Polymer Detection kit (ZYMED; Invitrogen) and VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA) or by Alexa Fluor 586 (red)-conjugated anti-mouse immunoglobulin G and Alexa Fluor 488 (green)-conjugated anti-rabbit immunoglobulin G (Molecular Probes; Invitrogen). Nuclear DNA was visualized by staining with 4',6-Diamidino-2'-phenylindole dihydrochloride (DAPI) (Roche Applied Science). Fluorescence was examined with an Axiophoto fluorescence microscope (Carl Zeiss MicroImaging, Thornwood, NY) and recorded

with a Zeiss Axiocam digital camera and image acquisition and analysis workstation. Immunostaining on the tissue microarray was performed similarly. They were analyzed by 2 investigators independently and classified as being very positive (++), positive (+), and negative (-). Concordant classification of the individual microarray spots was recorded, whereas those with discrepant classifications were reexamined by the 2 investigators and assigned with concordant classifications.

2.4. Statistical analysis

Correlation of gene expression changes between selected 2 genes was analyzed by Goodman and Kruskal's γ test [31]. Strength of correlation was scored by absolute value of γ ($|\gamma|$), range from 1 (perfect correlation) to 0 (absence of correlation).

3. Results

3.1. Initial immunohistochemical analysis of TSPY expression in HCC

To provide an initial assessment on TSPY expression in HCC, tissue microarrays containing duplicate spots of HCC, small numbers of related hepatocarcinomas and 20 normal liver samples were analyzed by immunohistochemistry with a TSPY-specific monoclonal antibody. Our results showed that 55 cases of HCC mostly Edmondson II/III grade and 5 cases of cholangiocarcinomas from male patients, tumors were stained positively for TSPY (Fig. 1A-D and F-I), representing 19.2% and 20.8%, respectively, of the corresponding male patient population (Table 1). All female samples and control normal hepatic tissues were negative for TSPY immunostaining. This preliminary study demonstrated that TSPY could be ectopically expressed in the tumor tissues of approximately 20% of male HCC patients but not female HCC specimens (Fig. 1E and J) nor normal liver tissues.

3.2. Association of TSPY expression and clinical and molecular biomarkers in HCC

To confirm our initial findings with tissue microarrays, a more detailed expression study was conducted with a panel of samples from patients with primary HCC, which consisted of high-quality RNAs derived from HCC tumors and adjacent nontumorous tissues, corresponding archival pathologic specimens for immunohistochemistry study and relevant clinical information. These patients included 32 males and 7 females with ages ranging from 29 to 82 years (male, 55.3 ± 10.1 years; female, 58.3 ± 17.7 years) (Table 2). Fourteen male patients (44%) and 2 female patients (29%) showed high serum AFP level ($\geq 200 \mu\text{g/L}$), and 8 male patients (25%) and one female patient (14%) showed extremely high serum AFP level ($\geq 2000 \mu\text{g/L}$).

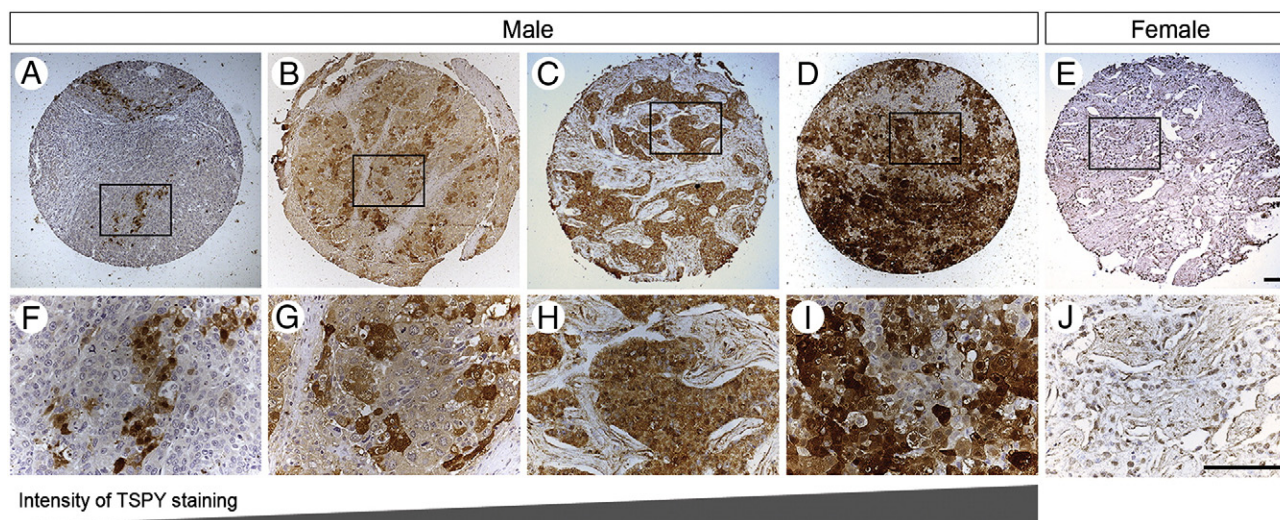


Fig. 1 Immunohistochemistry for TSPY expression in tissue microarrays of HCC and related tumors. A to D, Representative images of TSPY-positive male specimens with increasing signal intensities. F to I, Enlarged views of respective boxed areas in A to D. E and J, A female HCC sample, showing negative signals for TSPY. Bars represent 50 μ m.

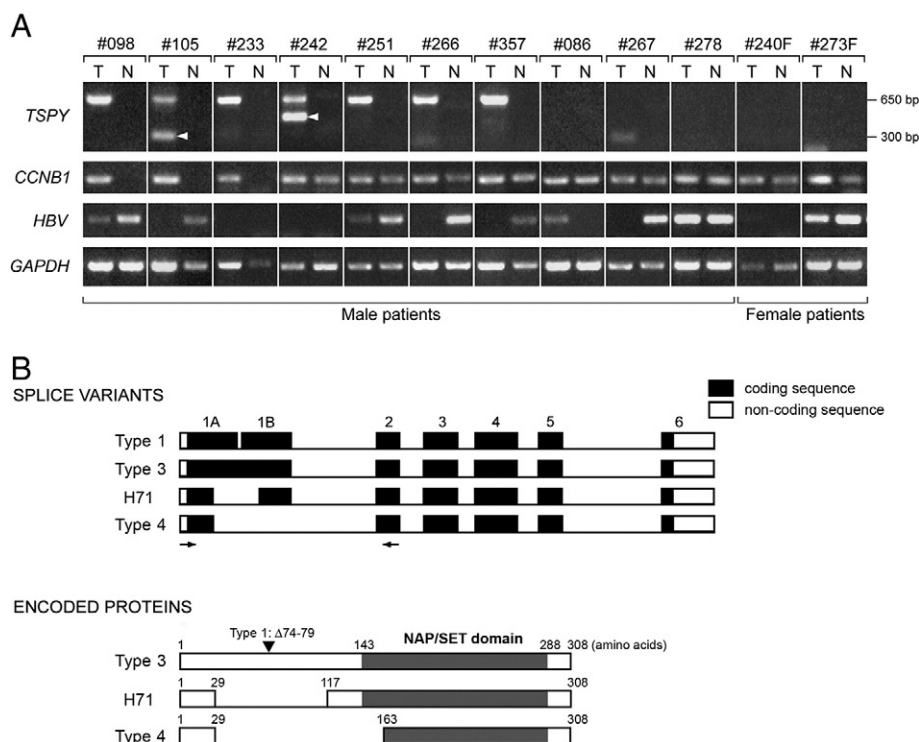


Fig. 2 Detection of *TSPY* transcripts in HCC pair-matched samples using RT-PCR strategy. A, Top panel shows representative results for *TSPY* expression in 12 independent pairs of HCC (T) and adjacent nontumorous liver (N) samples (10 males and 2 females). The predominant type 1 *TSPY* transcript was detected in 7 of 10 male HCC tumor samples but not in the corresponding nontumorous samples and female samples. Smaller bands representing short transcript variants were also detected in some cases (arrowheads). The expressions of *CCNB1* and HBV were similarly analyzed with respective transcript-specific primer sets. The RT-PCR of transcripts from the ubiquitously expressed *GAPDH* was used as a reference for RNA quality and positive control for each sample. B, Schematic illustration of splice variants of *TSPY* transcripts. Type 1, 3, 4, and H71 variants were detected by sequencing and analysis of DNA clones derived from the various RT-PCR products of *TSPY* transcripts detected in A. Type 4 is a novel splice variant, identified in the present study. Other variants have been described previously [12,32]. Arrows indicate the position of the forward and reverse primers used in the present RT-PCR study. The corresponding encoded proteins and the positions of the internal truncated are illustrated at the bottom panel of B. The truncation (H71 and type 4) and insertion (type 3) sites, represented by amino acid positions of the full-length type 1 protein, are as marked. The NAP/SET is a conserved domain between TSPY and TSPX and is relatively preserved among the encoded proteins, deduced from the open reading frames of the respective *TSPY* splice variants.

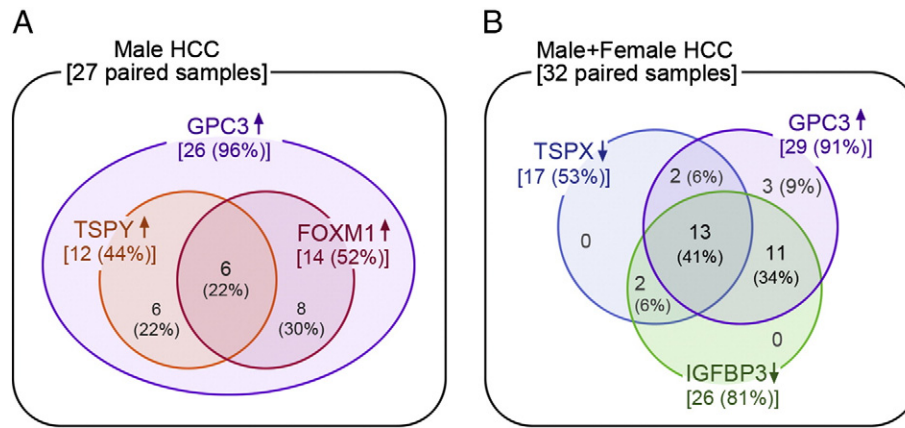


Fig. 3 A, Venn diagram showing the respective frequency of up-regulated expression of *GPC3*, *FOXM1*, and *TSPY* in HCC as compared with respective adjacent nontumorous liver samples in each pair-matched male samples (27 pairs). Cases with “not determined” value in Supplementary Table S1 are not counted. B, Venn diagram showing the respective frequency of up-regulated expression of *GPC3* and down-regulated expression of *TSPX* and *IGFBP3* in HCC as compared with corresponding nontumorous liver samples in each of the pair-matched male and female samples (32 pairs).

Histopathologically, 16 cases of male (52%) and 3 cases of female (43%) were classified to higher Edmondson grade (II-III) group. HBV-specific transcripts were detected by RT-PCR in 25 male patients (78%) and 4 female patients (57%).

To characterize the expression pattern of *TSPY* in HCC, RNA samples from 36 independent pairs of HCC (T) and adjacent nontumorous liver (N) samples were analyzed by RT-PCR. *TSPY* mRNA was expressed in 14 (48%) of 29 male samples, but no *TSPY* expression was detected in the corresponding adjacent nontumorous liver samples (Fig. 2A). In addition, 1 of 3 male HCC-alone samples (without adjacent nontumorous liver samples) was also positive for *TSPY* expression (case 269). Altogether, 15 (47%) of 32 male HCC samples showed *TSPY* expression. As references, the expression patterns of mitotic *CCNB1*, one of the *TSPY* binding partners [17] and transcripts for HBV and *GAPDH*, a glycolysis enzyme, were analyzed simultaneously. The results showed that *CCNB1* was differentially expressed in selected tumor and nontumorous tissue pairs (eg, 098, 105, and 233 in Fig. 2A) but mostly evenly in other sample pairs. The consistent expression of *CCNB1* in HCC specimens may suggest a potential interaction between *TSPY* and *CCNB1* in the *TSPY*-positive cases. HBV transcripts, however, showed more variability in its expression pattern between tumor and nontumorous tissue pairs, favoring either tumor or nontumorous tissue samples. *GAPDH* transcripts were consistently detected between tumor and nontumorous samples, despite some variations in intensities among a few paired samples (Fig. 2A).

Comparison of clinical profiles of the HCC patients with *TSPY* expression is summarized in Table 2. The results showed that *TSPY* was expressed more frequently (57%) among patients with high serum AFP (≥ 200 $\mu\text{g/L}$) or positive for HBV transcripts but relatively evenly among tumors of various Edmondson grades. Significantly, among those with extremely high serum AFP (ie, ≥ 2000 $\mu\text{g/L}$), such association reached a 75% level among HCC samples

positive for *TSPY*, suggesting that the expression of this Y-located protooncogene is closely associated with this clinical serum biomarker in HCC.

To further explore the correlation of the *TSPY* expression with additional biomarkers for HCC, we analyzed the expression of an HCC cancer cell biomarker, *GPC3* [21] and an oncogenic transcription factor, *FOXM1* [22,23] in both HCC and corresponding nontumorous liver tissues by conventional and qRT-PCR. Our results showed that *GPC3* was prominently up-regulated in 32 (89%) of the HCC samples, whereas *FOXM1* expression was elevated in 17 (47%) of the HCC tumor samples, respectively, as compared with the corresponding normal tissues (Supplementary Table S1). In the male patient group (total 27 paired samples except for cases with “not determined” value in Supplementary Table S1), up-regulation of *GPC3*, *FOXM1*, and *TSPY* in HCC was observed in 26 cases (96%), 14 cases (52%), and 12 cases (44%), respectively (Fig. 3A). Significantly, up-regulation of *TSPY* was observed only in the group with up-regulated *GPC3*. Its correlation with *FOXM1* was somewhat less robust, but 6 male cases (21%) showed up-regulation in expressions of all 3 investigated genes, *TSPY*, *GPC3*, and *FOXM1* (Fig. 3A). These data, although qualitative in nature, suggest that *TSPY* expression is closely associated with development of HCC cancer cells in which tumor biomarkers are up-regulated.

3.3. *TSPY* is colocalized with tumor biomarkers in HCC

To further investigate *TSPY* expression and localization in HCC specimens, we performed immunohistochemistry analysis using specific antibodies against *TSPY*, *GPC3*, and Ki-67 (a cell proliferative biomarker), respectively. Preliminary analysis showed that *TSPY* was readily detected in the tumor area of the HCC specimens (Fig. 4A and B), which were also positive for *GPC3* (Fig. 4C and D) and Ki-67

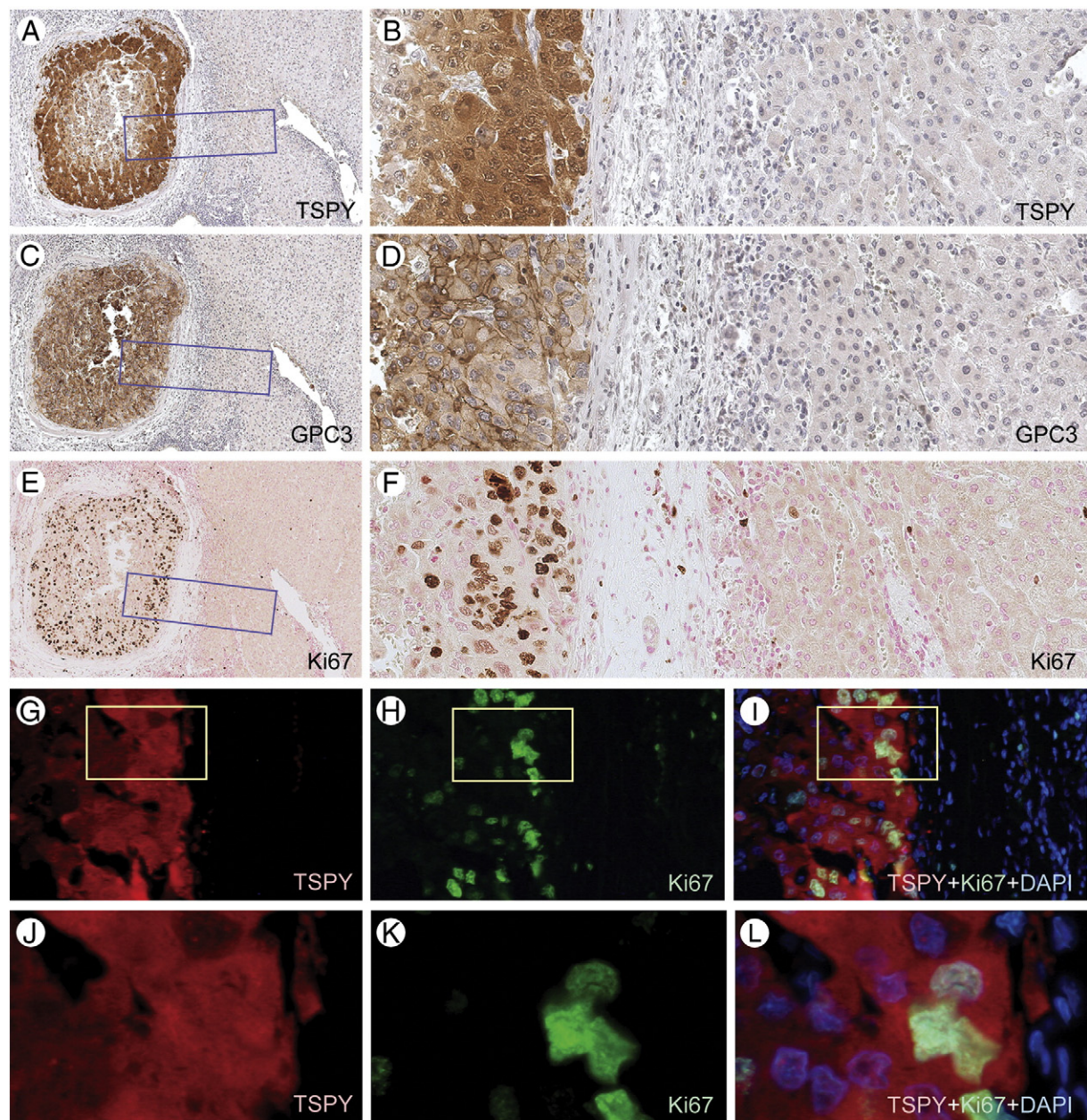


Fig. 4 Colocalization of TSPY, GPC3, and Ki-67 protein on tumor area of HCC. A to F, Immunostaining of TSPY (A), GPC3 (C), and Ki-67 (E) on adjacent sections of a male HCC specimen. Boxed areas in A, C, and E are enlarged in B, D, and F, respectively. G to L, Immunofluorescence of TSPY (G, red) and Ki-67 (H, green) and merged image (I) of G, H, and DAPI staining (blue). Boxed area in I is enlarged in J (TSPY), K (Ki-67), and L (merged), respectively. Bar represents 240 μm in A, C, and E; and 50 μm in B, D, F, G, H, and I.

(Fig. 4E and F). This distribution of TSPY-positive cells around the periphery of the tumor focus on this particular specimen correlated closely with that for cells positive for the cell proliferative biomarker, Ki-67. Indeed, immunofluorescence analysis confirmed the colocalization of TSPY and Ki-67 in these cells (Fig. 4G-L). Immunohistochemical analysis of sections from the corresponding specimens of the pair-matched samples corroborated the RT-PCR results, showing that tumor areas were positive for both TSPY

(Fig. 5A, C, E, and G) and GPC3 (Fig. 5B, D, F, and H) for RNA-positive samples for *TSPY* and *GPC3* (Fig. 2A and Supplementary Table S1). Conversely, male and female tumors, which were negative for *TSPY* transcripts, were also negative for TSPY protein (Fig. 5I and K, respectively), whereas *GPC3* expressions were detectable with qRT-PCR and faintly positive on immunostaining of adjacent sections (Fig. 5J and L, respectively). By and large, the intensities of immunostaining for TSPY proteins at the tumor areas were

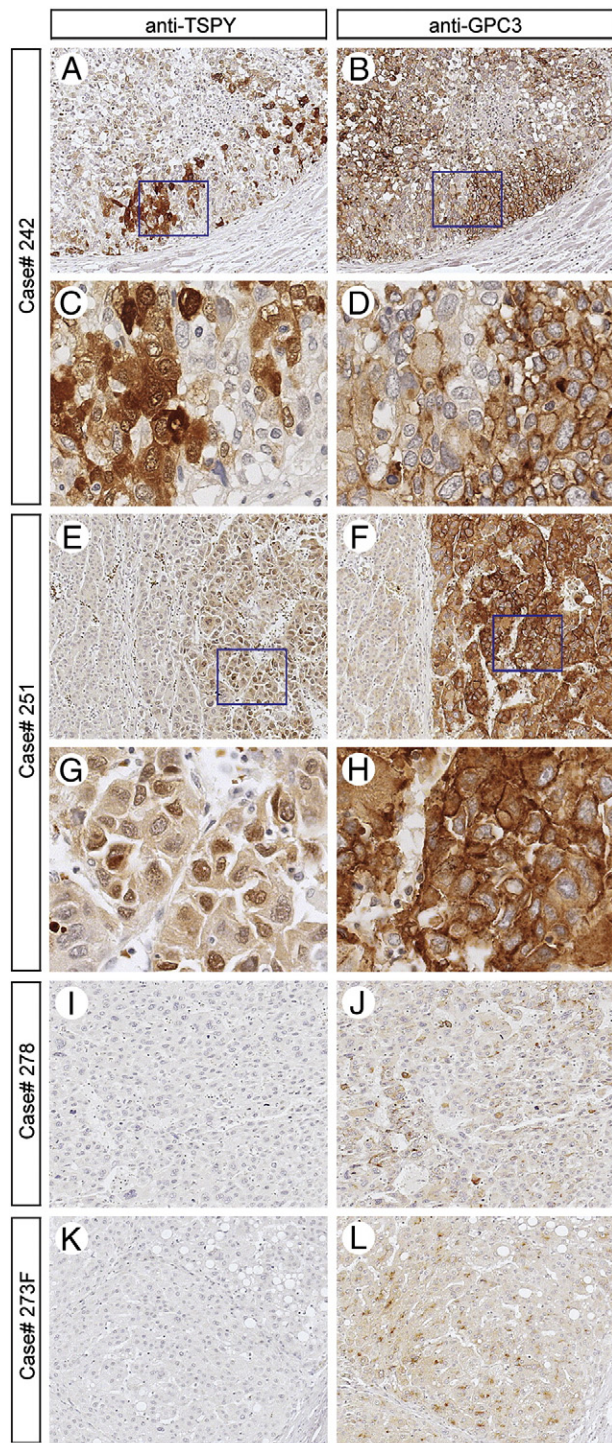


Fig. 5 Colocalization of TSPY and GPC3 expression in sections of pair-matched HCC samples for TSPY-positive cases 242 (A-D) and 251 (E-H) and TSPY-negative male case 278 (I-J) and female case 273F (K-L). Boxed areas in A, B, E, and F are enlarged in C, D, G, and H, respectively. Neither TSPY nor GPC3 was detected in the nontumorous area in the HCC tissue section from case 242 and 251. TSPY immunostaining was negative in case 278 and 273F but was faintly positive for GPC3 (confirmed by RT-PCR analysis). Bar represents 100 μ m in A and B, E and F, and I to L.

proportional to the relative levels of *TSPY* transcripts, as detected by conventional and qRT-PCR analyses of the corresponding RNAs (Figs. 2A and 6A).

3.4. Presence of TSPY variant transcripts capable of encoding various protein isoforms in HCC

Previous studies showed that *TSPY* transcriptional repeat units are capable of producing alternatively spliced transcripts harboring shortened but the same open reading frame as that of the major TSPY protein [12,32]. Most variant transcripts are generated from alternative/cryptic splice donor and acceptor sites between exons 1 and 2. Using a primer pair harboring these primary splice sites, multiple RT-PCR products were observed among HCC samples (eg, case 105 and case 242, white arrowheads in Fig. 2A). To determine the nature of these variant RT-PCR products, the *TSPY* bands were excised from the gel, cloned into a plasmid vector, and sequenced with an automatic sequencer. Sequence analysis indicated that the major bands at approximately 650 base pair (bp) size range (Fig. 2A) were derived from type 1 or type 3 *TSPY* variant transcripts [32]. The *TSPY* transcriptional unit harbors a small cryptic intron of 18 bp within exon 1, which separates the exon into exon 1A and 1B. Such a small intron is properly processed in the predominant type 1 transcript, which encodes the major 308-amino acid TSPY protein. Type 3 transcript skips such RNA splicing and produces a protein with 6 extra amino acids encoded by the 18-nucleotide cryptic intron in exon 1 (Fig. 2B). The splice variant H71 and type 4 transcripts (Fig. 2A, white arrowheads at 242 and 105, respectively) were generated by splicing events involving cryptic donor and acceptor sites within exon 1A and 1B and exon 1 and 2, respectively (Fig. 2B) [12,32], which produce abbreviated TSPY isoforms, containing in-frame deletions of 87 and 104 amino acids, respectively (Fig. 2B). These results suggest that a variety of splice variant transcripts of *TSPY* are expressed in HCC, as previously detected in prostate cancer and testicular germ cell tumors [12,32].

3.5. TSPX expression is repressed in HCC

As a putative tumor suppressor, capable of promoting the degradation of the HBV viral oncoprotein, HBx [20], retarding cell cycle progression, and activating p53 tumor suppressor [18,19], *TSPX* could serve an opposite role(s) against the tumor-promoting function(s) of its Y-homologue *TSPY* in HCC. Accordingly, examination of its expression in HCC could shed some insights on such probable function(s) for *TSPX*. To accomplish such objective, qRT-PCR analysis was performed with the same tumor and nontumorous tissue pairs (Fig. 6A and Supplementary Table S1). The results showed that TSPX was down-regulated in tumor region in 14 (54 %) of 26 male HCC cases and 4 (57 %) of 7 female HCC cases (Table 3). On the other hand, only 3 male cases of a

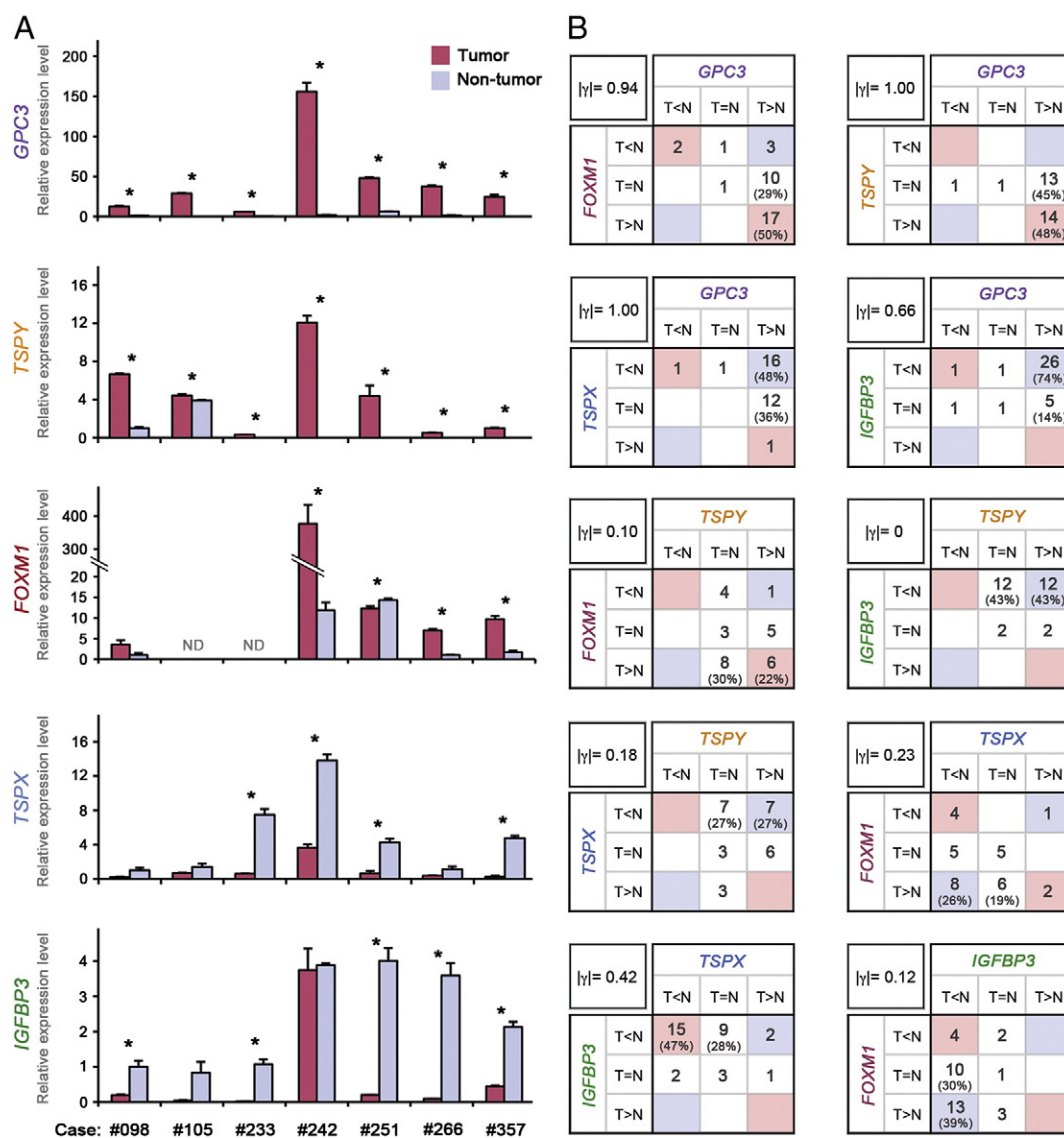


Fig. 6 A, Representative data of qRT-PCR analysis of 7 pair-matched tumor and nontumorous samples positive for *TSPY* expression and correlated with those for *GPC3*, *FOXM1*, *TSPX*, and *IGFBP3*. *indicates statistically significant difference in expression levels between respective tumor and nontumorous samples, $P \leq .01$. B, Correlation matrix analyses of *TSPY* and *TSPX* expression patterns with those of HCC biomarkers, *GPC3* and *FOXM1* and that of tumor suppressor, *IGFBP3*. $|\gamma|$ indicates the resultant value of Goodman and Kruskal's γ test for scoring the strength of association between 2 genes; range from 1 (strong association) to 0 (absence of association).

total 33 paired samples (9%) showed up-regulation of *TSPX* in tumor samples (Supplementary Table S1). Different from *TSPY*, there was no obvious correlation between down-regulation of *TSPX* and serum AFP level (Table 3). In addition to comparison with expressions of *TSPY*, *GPC3*, and *FOXM1*, a putative tumor suppressor, *IGFBP3* [24,25], was also analyzed in parallel. Our results showed that both *TSPX* and *IGFBP3* were consistently expressed at lower levels in selected tumor samples as compared with the respective adjacent normal tissues (Fig. 3B and Supplementary Table S1), especially among *TSPY*-positive male samples and were inversely correlated with expression patterns of *GPC3* (Fig. 6A).

3.6. Correlation of *TSPY* and *TSPX* with biomarkers in HCC

Using the correlation matrix, based on the relative levels of expression between tumor and adjacent nontumorous tissues, we had examined the relationships among the various biomarkers and statistically evaluated the strength of association by Goodman and Kruskal's γ test [31]; $|\gamma|$, range of value from 1 (strong association) to 0 (absence of association). The results indicate that the expression of *GPC3* strongly associated with that of *FOXM1* as expected ($|\gamma| = 0.94$), whereas the expression of *IGFBP3* negatively associated with that of *GPC3* ($|\gamma| = 0.66$).

Table 3 Correlation between TSPX expression and clinical parameters

	Male		Female	
	TSPX down-regulation (14 cases)	Total (26 cases)	TSPX down-regulation (4 case)	Total (7 cases)
Age	52.9 ± 12.7 (SD)	54.8 ± 10.8 (SD)	54.3 ± 20.0 (SD)	58.3 ± 17.7 (SD)
Edmondson grading ^a				
I/II	6 (46%) ^c	13	2 (50%) ^c	4
II/III	7 (58%) ^c	12	2 (67%) ^c	3
Serum AFP				
<200 µg/L	6 (43%) ^c	14	3 (60%) ^c	5
≥200 µg/L	8 (67%) ^c	12	1 (50%) ^c	2
<2000 µg/L	11 (58%) ^c	19	3 (50%) ^c	6
≥2000 µg/L	3 (43%) ^c	7	1 (100%) ^c	1
HBV transcript ^b				
HBV RNA negative	5 (100%) ^c	5	2 (67%) ^c	3
HBV RNA positive	9 (43%) ^c	21	2 (50%) ^c	4

^a One case was not graded or measured.^b HBV RNA expression was analyzed by RT-PCR.^c Percent of total cases in the respective category.

Likewise, *TSPY* expression is closely associated with *GPC3* expression ($|\gamma| = 1$) and, to a much lesser extent, that of *FOXMI* ($|\gamma| = 0.10$). The situation is reversed for its X homologue, *TSPX*, which is modestly associated with *IGFBP3* expression ($|\gamma| = 0.42$) and inversely with those of *GPC3* ($|\gamma| = 1$) and *FOXMI* ($|\gamma| = 0.23$) (Fig. 6B). No association was observed between *TSPY* expression and *IGFBP3* expression ($|\gamma| = 0$). These observations suggest that *TSPY* is a male-specific protooncogene on the Y chromosome, whose ectopic expression could contribute to the oncogenic processes in a male-biased manner, whereas its X homologue, *TSPX*, is an X-linked tumor suppressor, whose expression could likely oppose the *TSPY* actions and suppress HCC initiation and/or progression.

4. Discussion

In the present study, we demonstrated that *TSPY* expression was closely correlated with established tumor biomarkers, such as *GPC3* and to a lesser extent *FOXMI* (Fig. 6 and Supplementary Table S1), and its protein was colocalized in tumor cells with *GPC3* and the proliferative cell marker, Ki-67 (Figs. 4 and 5). *GPC3* is an important X-linked cell surface protein highly expressed in HCC and other solid tumors [21,33,34]. *FOXMI* is considered to be a master regulator of tumor metastasis, particularly in HCC. Overexpression of *FOXMI* is associated with aggressive tumor features and poor prognosis among HCC patients [22,23]. In contrast, the expression of *TSPX*, the X homologue of *TSPY*, inversely correlated with those of *GPC3* and *FOXMI* but directly correlated with that for a tumor suppressor, *IGFBP3*, in HCC. *IGFBP3* is a key high-affinity binding protein to insulin-like growth factors, which

play critical roles in the initial oncogenesis of HCC [24,25]. *IGFBP3* and other insulin-like growth factor binding proteins modulate the bioavailability of insulin-like growth factors (IGFs). These observations suggest that this pair of homologous genes, that is, *TSPY* and *TSPX*, on the sex chromosomes possess contrasting properties and likely serving opposing functions in hepatocarcinogenesis.

What are the likely mechanisms by which *TSPY* and *TSPX* exert their respective functions in HCC development? As the putative gene for the gonadoblastoma locus on the Y chromosome locus, *TSPY* is postulated to serve a normal function in the testis but becomes oncogenic when it is inappropriately expressed in dysfunctional germ cells, such as those in dysgenetic gonads of patients with disorders of sex development, who are significantly at risk (>66%) in developing gonadoblastoma, a benign form(s) of germ cell tumors [4,13]. We have previously showed that *TSPY* binds to type B cyclins and stimulates the activities of an activated cyclin B-cyclin dependent kinase 1 (CDK1) complex [17], essential for 2 successive rounds of meiotic division during spermatogenesis [10]. *TSPY* becomes oncogenic when it is inappropriately expressed in dysfunctional germ cells, as discussed above, or somatic cells, such as impaired hepatocytes incapable of entering meiosis, but instead it promotes cell proliferation and a rapid transition of the G₂/M phase [35], via its actions on the mitotic cyclin B-CDK1 kinase activities in this phase of the cell cycle [17]. Furthermore, *TSPY* could indirectly exert additional oncogenic functions by up-regulating various oncogenes and growth promoters, particularly those residing on the human chromosome 12p, including *FOXMI*, and down-regulating tumor suppressors, including *IGFBP3*, and growth inhibitors [12,35]. Importantly, as demonstrated before, *TSPY* frequently produces abbreviated transcripts with in-frame deletion of specific regions of the predominant protein in

various tumorigenic tissues, such as testicular germ cell tumors [12], prostate cancer [32], and HCC, in the present study. These internally truncated TSPY proteins are more potent than the full-length protein in accelerating cell cycle progression and colony formation in various transfection assays (Oram and Lau, unpublished observations). Hence, the Y-located *TSPY* gene could act as a protooncogene and collectively favor cancer development or progression in the host cells in a male-specific manner.

As a homologue of *TSPY* on the X chromosome, *TSPX* originated from the same ancestral gene during the evolution of the mammalian sex chromosomes [16]. TSPY and TSPX harbor a conserved nucleosome assembly protein/SET nuclear oncoprotein (NAP/SET) domain [5,16] but diverge considerably in the flanking regions of their proteins, especially the acidic domain at the carboxyl terminus of TSPX, which is absent in TSPY. Several studies demonstrated that overexpression of TSPX retards cell cycle progression and stabilizes the signaling pathway of the tumor suppressor p53 [18,19]. TSPX could also bind to cyclin B–CDK1 complex but repress its kinase activities, specifically via its carboxyl acidic domain [17]. Hence, TSPX could function as a G₂/M checkpoint important in modulating a proper transition of this stage of the cell cycle. More significantly, TSPX is capable of promoting a proteasomal degradation of HBx oncoprotein encoded by the genome of HBV, a major HCC pathogenic agent [7,8,20]. Hence, its functions in viral protein degradation, cell cycle modulation, and p53 stabilization suggest that *TSPX* is an X-linked tumor suppressor in HCC development and/or progression.

In summary, the present data clearly suggest that ectopic activation of *TSPY* and/or genetic mutation/epigenetic silencing of *TSPX* could collectively contribute to the complex etiologies of liver cancers in male-biased manners. Further studies with additional panels of tumor and nontumorous sample pairs should provide better correlations between TSPY/TSPX and other biomarkers of HCC including GPC3, FOXM1, and HBV/HCV viral and environmental risk factors.

Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.humpath.2014.05.002>.

References

- [1] Baggio G, Corsini A, Floreani A, Giannini S, Zagonel V. Gender medicine: a task for the third millennium. *Clin Chem Lab Med* 2013;51:713-27.
- [2] Dorak MT, Karpuzoglu E. Gender differences in cancer susceptibility: an inadequately addressed issue. *Front Genet* 2012;3:268.
- [3] Liu R, Kain M, Wang L. Inactivation of X-linked tumor suppressor genes in human cancer. *Future Oncol* 2012;8:463-81.
- [4] Lau YF. Gonadoblastoma, testicular and prostate cancers, and the TSPY gene. *Am J Hum Genet* 1999;64:921-7.
- [5] Lau YF, Li Y, Kido T. Gonadoblastoma locus and the TSPY gene on the human Y chromosome. *Birth Defects Res C Embryo Today* 2009;87:114-22.
- [6] Bosch FX, Ribes J, Cleries R, Diaz M. Epidemiology of hepatocellular carcinoma. *Clin Liver Dis* 2005;9:191-211.
- [7] Hernandez-Gea V, Toffanin S, Friedman SL, Llovet JM. Role of the microenvironment in the pathogenesis and treatment of hepatocellular carcinoma. *Gastroenterology* 2013;144:512-27.
- [8] Kew MC. Epidemiology of chronic hepatitis B virus infection, hepatocellular carcinoma, and hepatitis B virus-induced hepatocellular carcinoma. *Pathol Biol (Paris)* 2010;58:273-7.
- [9] Zhang JS, Yang-Feng TL, Muller U, Mohandas TK, de Jong PJ, Lau YF. Molecular isolation and characterization of an expressed gene from the human Y chromosome. *Hum Mol Genet* 1992;1:717-26.
- [10] Lau YF, Li Y, Kido T. Role of the Y-located putative gonadoblastoma gene in human spermatogenesis. *Syst Biol Reprod Med* 2011;57:27-34.
- [11] Lau Y, Chou P, Iezzoni J, Alonzo J, Komuves L. Expression of a candidate gene for the gonadoblastoma locus in gonadoblastoma and testicular seminoma. *Cytogenet Cell Genet* 2000;91:160-4.
- [12] Li Y, Tabatabai ZL, Lee TL, et al. The Y-encoded TSPY protein: a significant marker potentially plays a role in the pathogenesis of testicular germ cell tumors. *HUM PATHOL* 2007;38:1470-81.
- [13] Li Y, Vilain E, Conte F, Rajpert-De Meyts E, Lau YF. Testis-specific protein Y-encoded gene is expressed in early and late stages of gonadoblastoma and testicular carcinoma in situ. *Urol Oncol* 2007;25:141-6.
- [14] Gallagher WM, Bergin OE, Rafferty M, et al. Multiple markers for melanoma progression regulated by DNA methylation: insights from transcriptomic studies. *Carcinogenesis* 2005;26:1856-67.
- [15] Yin YH, Li YY, Qiao H, et al. TSPY is a cancer testis antigen expressed in human hepatocellular carcinoma. *Br J Cancer* 2005;93:458-63.
- [16] Lau YF, Kido T, Li Y. The TSPY gene family. In: Lau YF, Chan WY, editors. *The Y chromosome and male germ cell biology in health and diseases*. Hackensack: World Scientific Publishing Co. Pte. Ltd.; 2007. p. 73-90.
- [17] Li Y, Lau YF. TSPY and its X-encoded homologue interact with cyclin B but exert contrasting functions on cyclin-dependent kinase 1 activities. *Oncogene* 2008;27:6141-50.
- [18] Kandalaf LE, Zudaire E, Portal-Nunez S, Cuttitta F, Jakowlew SB. Differentially expressed nucleolar transforming growth factor-beta1 target (DENTT) exhibits an inhibitory role on tumorigenesis. *Carcinogenesis* 2008;29:1282-9.
- [19] Tu Y, Wu W, Wu T, et al. Antiproliferative autoantigen CDA1 transcriptionally up-regulates p21(Waf1/Cip1) by activating p53 and MEK/ERK1/2 MAPK pathways. *J Biol Chem* 2007;282:11722-31.
- [20] Kido T, Ou JH, Lau YF. The X-linked tumor suppressor TSPX interacts and promotes degradation of the hepatitis B viral protein HBx via the proteasome pathway. *PLoS One* 2011;6:e22979.
- [21] Shirakawa H, Kuronuma T, Nishimura Y, et al. Glypican-3 is a useful diagnostic marker for a component of hepatocellular carcinoma in human liver cancer. *Int J Oncol* 2009;34:649-56.
- [22] Koo CY, Muir KW, Lam EW. FOXM1: from cancer initiation to progression and treatment. *Biochim Biophys Acta* 1819;2012:28-37.
- [23] Sun H, Teng M, Liu J, et al. FOXM1 expression predicts the prognosis in hepatocellular carcinoma patients after orthotopic liver transplantation combined with the Milan criteria. *Cancer Lett* 2011;306:214-22.
- [24] Regel I, Eichenmuller M, Joppin S, et al. IGFBP3 impedes aggressive growth of pediatric liver cancer and is epigenetically silenced in vascular invasive and metastatic tumors. *Mol Cancer* 2012;11:9.
- [25] Subramaniam K, Ooi LL, Hui KM. Transcriptional down-regulation of IGFBP-3 in human hepatocellular carcinoma cells is mediated by the

- binding of TIA-1 to its AT-rich element in the 3'-untranslated region. *Cancer Lett* 2010;297:259-68.
- [26] Kido T, Lau YF. A Cre gene directed by a human TSPY promoter is specific for germ cells and neurons. *Genesis* 2005;42:263-75.
- [27] Kido T, Hatakeyama S, Ohyama C, et al. Expression of the Y-encoded TSPY is associated with progression of prostate cancer. *Genes (Basel)* 2010;1:283-93.
- [28] Chan CF, Yau TO, Jin DY, Wong CM, Fan ST, Ng IO. Evaluation of nuclear factor-kappaB, urokinase-type plasminogen activator, and HBx and their clinicopathological significance in hepatocellular carcinoma. *Clin Cancer Res* 2004;10:4140-9.
- [29] Lau YF, Zhang J. Expression analysis of thirty one Y chromosome genes in human prostate cancer. *Mol Carcinog* 2000;27:308-21.
- [30] Kido T, Lau YF. The human Y-encoded testis-specific protein interacts functionally with eukaryotic translation elongation factor eEF1A, a putative oncoprotein. *Int J Cancer* 2008;123:1573-85.
- [31] Kianifard F, Chen M. Extending Goodman-Kruskal's gamma to the comparison of ordered treatments in multicenter clinical trials. *J Biopharm Stat* 1999;9:521-9.
- [32] Lau YF, Lau HW, Komuves LG. Expression pattern of a gonadoblastoma candidate gene suggests a role of the Y chromosome in prostate cancer. *Cytogenet Genome Res* 2003;101:250-60.
- [33] Hav M, De Potter A, Ferdinande L, et al. Glypican-3 is a marker for solid pseudopapillary neoplasm of the pancreas. *Histopathology* 2011;59:1278-9.
- [34] Zynger DL, McCallum JC, Luan C, Chou PM, Yang XJ. Glypican 3 has a higher sensitivity than alpha-fetoprotein for testicular and ovarian yolk sac tumour: immunohistochemical investigation with analysis of histological growth patterns. *Histopathology* 2010;56:750-7.
- [35] Oram SW, Liu XX, Lee TL, Chan WY, Lau YF. TSPY potentiates cell proliferation and tumorigenesis by promoting cell cycle progression in HeLa and NIH3T3 cells. *BMC Cancer* 2006;6:154.