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# The Y-Encoded TSPY Protein: a Significant Marker Potentially Plays a Role in the Pathogenesis of Testicular Germ Cell Tumors

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## Summary

The testis-specific protein Y-encoded (TSPY) gene is the putative gene for the gonadoblastoma locus on the Y chromosome (GBY) that predisposes dysgenetic gonads of intersex patients to gonadoblastoma development. TSPY is expressed at high levels in gonadoblastoma tissues, supporting its possible oncogenic function in this type of germ cell tumors. To explore the possibility that this Y chromosome gene is also involved in pathogenesis of the more common testicular germ cell tumors (TGCTs), we have conducted various expression studies using immunohistochemistry, Western blotting and RT-PCR analysis on 171 cases of TGCTs and selected normal testis controls. Our results demonstrated that TSPY protein is abundantly expressed in the precursor, carcinoma-in-situ or intratubular germ cell neoplasia unclassified (CIS/ITGCNU), and seminoma, but only minimally or not expressed in various types of nonseminomas. TSPY co-expresses with established germ cell tumor markers, such as PLAP, c-KIT, OCT4 and proliferative markers, such as Ki-67 and cyclin B1, in the same tumor cells at both RNA and protein levels. Ectopic TSPY expression in cultured cells up regulates pro-growth genes, including those at chromosome 12p13, frequently gained/amplified in TGCTs. Our results suggest that TSPY, in combination with other markers, could be an important marker for diagnosis and subclassification of TGCTs and support its role in the pathogenesis of both gonadoblastoma and TGCTs.

### Keywords

gonadoblastoma locus; Y chromosome; TSPY; diagnostic marker; 12p genes; testicular germ cell tumors

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## 1. Introduction

The testis-specific protein Y-encoded (TSPY) gene is a tandemly repeated gene on the short arm of the human Y chromosome [1–3]. Most of its functional transcriptional units have been mapped within the critical region harboring the gonadoblastoma locus on the Y chromosome (GBY) [4–6], the only oncogenic locus on this male-specific chromosome. GBY is hypothesized to serve a normal physiological function in germ cell proliferation and/or differentiation, but could predispose incompatible germ cells, e.g. those in an ovarian environment or dysfunctional/dysgenetic testis, to tumorigenesis [4,7]. Gonadoblastoma arises most frequently in dysgenetic gonads of XY females, intersex individuals and, to a lesser extent, Turner patients with residual Y chromosome materials [8–12]. Recent studies demonstrated that TSPY sequences are indeed present in the genomes of these XY females and intersex individuals and are expressed abundantly in this special type of germ cell tumors [13–15], thereby supporting the candidacy of TSPY as the gene for GBY.

Several studies have documented the expression of TSPY in the more common forms of testicular germ cell tumors (TGCTs) of adult testis, classified as seminomas and nonseminomas [2,7,16–18]. However, its value as a diagnostic marker for subtypes of these prevalent cancers among young men between the ages of 15 to 35 years old has not been established. At present, a detailed investigation regarding its colocalization with other known markers has not been performed so far. Further, various isoforms of TSPY transcripts and proteins have been demonstrated in cancerous samples [16]. It is uncertain if such isoforms exist in various types of TGCTs. To address these questions, we have conducted a comprehensive study in establishing its expression pattern with reference to those of other germ cell tumor markers, such as OCT4, c-KIT and placental-like alkaline phosphatase (PLAP), alpha fetal protein (AFP), and human chorionic gonadotrophin (hCG) [19]. Our results showed that TSPY is expressed predominantly in testicular seminoma and the precursor, carcinoma-in-situ (CIS) or intratubular germ cell neoplasia unclassified (ITGCNU), but not in various types of nonseminomas, including embryonal carcinoma, teratoma, choriocarcinoma and yolk sac tumors. Its expression pattern parallels those of OCT4, c-KIT and PLAP in CIS/ITGCNU and seminomas, but not AFP and hCG in nonseminomas. The differential expression pattern of TSPY in seminomatous and nonseminomatous germ cell tumors suggests that it can be used as a diagnostic marker for detection of precursors of germ cell tumors and for subtyping of TGCTs. Hence, TSPY, in combination with other markers, could be an important marker for diagnosis and subclassification of TGCTs.

TGCTs are postulated to originate from the CIS/ITGCNU precursor. Currently the mechanism (s) by which this pre-malignant precursor initiates and develops into both seminomas and nonseminomas is unknown. Various genetic studies have demonstrated that CIS/ITGCNU and TGCTs are aneuploid. A gain of complete short arm of chromosome 12, and sometimes amplification of certain portion of it, has been the consistent change(s) in the evolving germ cell tumor genome [19–22]. Such gain of chromosome 12p genes seems to be associated with advancement of the oncogenic process(es) and increase in pluripotency of the tumor cells [20,21,23,24]. Previously, we have demonstrated that ectopic TSPY expression in cultured cells up regulates pro-growth genes, including those at chromosome 12p13, and accelerates the $G_2$/M transition in the cell cycle [25]. Analysis of available microarray data demonstrates a correlation between TSPY expression and up-regulation of certain chromosome 12p13 genes in clinical CIS/ITGCNU and TGCT samples [26,27]. The cell cycle regulatory function(s) of TSPY and the present results, therefore, support a role(s) for this Y chromosome gene in the pathogenesis of both gonadoblastoma and TGCTs.

## 2. Materials and Methods

### 2.1. Tissue Collection

A total of 171 testicular germ cell tumors consisting of 86 seminomas and 85 nonseminomas and 17 normal testicular tissues were analyzed in the present study. They were obtained from Department of Urology, Hirosaki University School of Medicine, Cooperative Human Tissue Network, and Department of Pathology, Veterans Affairs Medical Center, San Francisco. Except two cases of nonseminomas at ages of 4 and 9 years, most TGCT patients were between 19 to 68 years. Of which, 131 cases with clinical staging information from the Department of Urology, Hirosaki University, Japan, were used in correlating the TSPY expression with clinical staging using a crude scoring system, as described in the Results section. Of these 131 cases, 61 cases were seminomas and 70 cases were nonseminomas consisting of embryonal carcinoma, yolk sac tumor, teratoma, choriocarcinoma and mixtures thereof. The median ages for the seminoma and nonseminoma groups were 34 and 27.5 years respectively. Patients with mixed germ cell tumors harboring nonseminomatous and seminomatous components were considered as nonseminomas. The tumor type classification was selectively examined and confirmed by a pathologist at the VA Medical Center. The seminoma and CIS/ITGCNU components were excluded in these nonseminoma specimens and were studied separately in our scoring analysis. Frozen samples of TGCTs were obtained from the Cooperative Human Tissue Network and used for protein and RNA analyses. The classification of these frozen samples were based on the pathologist reports provided from CHTN. Additional protein and RNA samples from various TGCTs were provided by Professor Leendert Looijenga, Erasmus Medical Center, Rotterdam, Netherlands. They have previously been characterized by immunostaining with specific TSPY antibody. The presence of TSPY RNA and proteins in these samples corresponded to the immunohistochemistry results. Tissue arrays containing normal fetal and adult testes were purchased from Cybrdi Inc. (Frederick, MD). All studies of human archival pathological specimens were performed under a protocol approved by the Institutional Committee on Human Research, VA Medical Center, San Francisco.

### 2.2. Antibodies

The mouse monoclonal antibodies (#2 and #7) against recombinant TSPY were generated in our laboratory [28]. Other antibodies were obtained from the following commercial sources: 1) Lab Vision (Fremont, CA), a rabbit polyclonal antibody against PLAP (SP15, RM-9115), a rabbit monoclonal antibody against Ki-67 (Clone SP6, RM-9106), a rabbit antibody against the alpha fetal protein (AFP) (RB9064), a mouse monoclonal antibody against the human chorionic gonadotrophin (SPM105); 2) Dako Corp. (Carpinteria, CA), a rabbit polyclonal antibody against c-KIT and a mouse monoclonal antibody against human cyclin B1 (A4502); 3) Sigma Biochemicals (St. Louis, MO), a mouse monoclonal antibody against tubulin; and 4) Santa Cruz Biotechnology Inc. (Santa Cruz, CA), a goat polyclonal antibody against OCT4 (C-20, sc-8629). The OCT4 antibody has been demonstrated to be informative for immunohistochemistry and Western blotting of TGCTs [29].

### 2.3. Immunohistochemistry and immunofluorescence

Immunohistochemical staining was performed as previously described [28]. Briefly, formalin-fixed and paraffin-embedded TGCT tissue sections were deparaffinized with xylene, and hydrated with an ethanol-water series. Antigen retrieval was performed by incubating the slides in 50mM Tris-HCl pH10 at 95°C for 20–30 min. Endogenous peroxidase was inactivated in 3% $H_2O_2$ for 15 min at room temperature. The sections were blocked with 3% BSA, 0.1% Triton X-100 in PBS for 1 hour and were incubated overnight at 4°C with the respective primary antibodies, at dilution ratios of 1:1000 for TPSY monoclonal antibody, 1:50 for PLAP rabbit antibody, 1:500 for c-KIT rabbit antibody, 1:500 for OCT4 goat antibody, 1:200 for AFP antibody, 1:50 for hCG antibody and 1:100 for Ki-67 rabbit monoclonal antibody. The primary

antibodies were then detected with corresponding biotinylated secondary antibodies and visualized by avidin–biotin detection and substrate kits (Vector Laboratories, Burlingame, CA). The immunostained sections were reviewed independently by at least two investigators. For immunofluorescence double staining, sections were processed similarly as above, the signals were visualized with FITC or Alexas 594 conjugated secondary antibodies (Cell Signaling, Danvers, MA) and fluorescence microscopy. Normal testis sections were used as positive control for TSPY. As negative controls, parallel sections were processed similarly without the respective primary antibody for each immunostaining experiment.

### 2.4. RT-PCR Analysis

Frozen TGCT tissues were obtained from the Cooperative Human Tissue Network. Total RNAs were isolated from these frozen tissues using TRIzol reagents (Invitrogen, Carlsbad, California). After treatment with RNase-free DNase (Promega, Madison, WI), 2 μg of treated total RNA were used for cDNA synthesis with SuperScripts III Kit (Invitrogen, Carlsbad, CA) in a 20 μl reverse transcriptase reaction mixture. PCR was performed with 1 μl of each of the cDNA reactions with specific primer sets and a touchdown protocol [16]. Semi-quantitative RT-PCR and estimation of amplified products were performed as previously described [25]. GAPDH and HPRT were used as controls in respective experiments, as noted.

### 2.5. Western Blot Analyses

Western blotting was performed with established procedures [28,30]. Frozen tissues were homogenized in lysis buffer (50mM Tris-HCl pH7.5, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100) with a polytron, and centrifuge at 16000g for 10 min. The protein concentration was determined by a Bio-Rad DC protein assay kit (Bio-RAD, Richmond, CA). One hundred μg of each lysate were resolved on 10% SDS-PAGE, and transferred onto nitrocellulose membranes by electroblotting. Human HEK293 cells were transfected individually with expression vectors for the cDNAs of various TSPY isoforms under the CMV promoter and analyzed similarly after 48 hours by Western blotting [28,30]. The membranes were incubated with various primary antibodies at various dilutions, as above. The signals were visualized with respective horseradish peroxidase-conjugated secondary antibodies and ECL plus chemiluminescence kit (GE Healthcare, Piscataway, NJ). The filters were stripped and re-probed or parallel ones were probed with additional antibodies and the signals detected similarly.

### 2.6 Hierarchical clustering of gene expression data

Gene expression data of selected number genes mapped to chromosome 12 band 13 were obtained from GEO database [26]. The GEO datasets were: normal testes (NT1=GSM31729; NT2=GSM31728; NT3=GSM31803), CIS/ITGCNU samples (ITGCNU1=GSM33594; ITGCNU2=GSM31730; ITGCNU3=GSM31731), seminomas (SE1=GSM33595; SE2=GSM31732; SE3=GSM33942), and embryonal carcinoma (EC1=GSM33944). Other microarray datasets were derived from published results [27]. The expression data in $\log_2$ ratios were analyzed by a one-sample *t*-test to detect significant differences in gene expression. Genes that showed differential expression with *P* values of $\leq 0{\cdot}01$ were considered statistically significant. Normalized gene expression ratios between samples were analyzed using the significant analyses for microarray (SAM) algorithm with less than 5% false discovery rate (FDR). The expression profiles resulting SAM analysis were grouped based on similarity in pattern of their expression by using hierarchical cluster analysis based on the Pearson correlation by TIGR MultiExperiment Viewer software version 4.01 [31].

## 3. Results

### 3.1. TSPY is Preferentially Expressed in Seminomas and Germ Cell Tumor Precursors

To correlate the TSPY expression at the early stage of TGCTs, we have examined a total of 171 cases of TGCTs harboring various features, including CIS/ITGCN, seminoma, yolk sac tumor, embryonal carcinoma and choriocarcinoma, using immunostaining techniques. Our results showed that TSPY was preferentially expressed at high levels in CIS/ITGCNU and seminoma specimens (Figure 1A–E). Such immunostaining persisted in metastatic seminoma cells in the lymph node (Figure 1F, J). However, immunostaining signals for TSPY were at minimal or negative levels in nonseminomas, including teratoma, yolk sac tumors (Figure 1H) and embryonal carcinomas (Figure 1G, I, N and O). Selected areas of the yolk sac tumors and embryonal carcinomas might contain clusters of CIS/ITGCNU whose tumor cells are highly positive for TSPY (Figure 1G–I, K–M, N–P), as those in seminoma samples (Figure 1B, C).

### 3.2. TSPY is Co-Expressed with Established Tumor Markers for Seminoma and Carcinoma-in-situ/Intratubular Germ Cell Neoplasia Unclassified

The preferential expression of TSPY in testicular seminoma and CIS/ITGCNU suggests that this GBY candidate gene could serve as a specific tumor marker for these types of germ cell tumors. To determine its expression pattern in reference to other established germ cell tumor markers, such as PLAP, OCT4, c-KIT and the proliferative marker, Ki-67, we had performed double immunofluorescence analysis on selected testicular seminoma and CIS/ITGCNU specimens. Our results demonstrated that TSPY was co-expressed in the same tumor germ cells of both types of TGCTs, despite variation of their subcellular locations and heterogeneity in staining intensity (Figure 2). TSPY was located in both cytoplasm and nuclei of the tumor germ cells. PLAP and c-KIT (the tyrosine kinase receptor for stem cell factor) were primarily located on the cell surface while OCT4 (the stem cell transcription factor) and Ki-67 (the proliferative marker) were located on the nuclei [32]. Similar to TSPY, cyclin B1 (the mitotic cyclin) could be located on both cytoplasm and nuclei. All other germ cell tumor markers co-expressed mostly with TSPY protein in the same tumor germ cells, as revealed by merged images of the respective tissue sections (Figure 2C, F, I, L, O, R & U), except the proliferative marker (Ki-67) that was only highly expressed in selected TSPY positive cells (Figure 2X).

### 3.3. Variant TSPY Proteins are Expressed in both Normal and Tumor Germ Cells

To confirm our immunostaining results, selected cases of seminomas, nonseminomas and normal testes were analyzed with Western blotting (Figure 3A). Our results showed that TSPY was indeed expressed at high levels in seminoma samples, but minimally in mixed TGCT, and embryonal carcinomas. Normal testes showed reduced but detectable levels (Figure 3A, lanes 1–3, 11). Interestingly, multiple bands were observed in both seminomas and normal testis samples (e.g. Figure 3A, lanes 1–9). These bands correspond to those expressed in HEK293 cells transfected with expression vectors for respective isoforms of TSPY proteins (Figure 3A, left 3 lanes) [16]. Reprobing of the same filters with various specific antibodies showed that specific TGCT markers, such as PLAP, c-KIT and OCT4, were highly expressed in seminoma samples, but not in normal testes. Significantly, both OCT4 and Cyclin B1 showed similar high levels of expression in the embryonal carcinoma samples as those of the seminomas. We also observed similar immunostaining intensity for these two markers in the same specimens.

Various TSPY isoforms are generated by alternative splicing events of transcripts originating from the same transcriptional units [16,33]. In general, these variant transcripts can be classified into two categories. The first one utilizes a cryptic donor site at codon #29 and acceptor sites at codon #117, 134 and 169, and generates three variant transcripts, designated as Exon1A, 1B and 1C. They encode abbreviated TSPY isoforms harboring in-frame deletions of 87, 104 and 139 amino acids respectively. The second category of transcripts involves intron skipping

events, thereby introns 3 or 4 could be included in the final variant transcripts. They encode variant TSPY proteins harboring a normal amino end but diverge at the intron junctions with short carboxyl termini different from those of the majority of the isoforms (Figure 3C) [16]. To confirm the existence of these variant transcripts and the isoforms detected by Western blot, RT-PCR analysis with transcript-specific primer sets (Table 1) was conducted with total RNAs derived from the same samples, used in the Western blotting (Figure 3A). Our results showed that these variant transcripts were present in these specimens. Although semi-quantitative in nature, the relative amounts of RT-PCR products (Figure 3B) were similar to those of proteins detected by Western blotting in the respective samples (Figure 3A). Similar results were obtained for the transcripts for the various germ cell tumor markers, cyclin B1 and controls (Figure 3B).

### 3.4. TSPY is an Excellent Diagnostic Marker for TGCTs

The specific expression of TSPY in CIS/ITGCNU and seminoma clearly suggests that this protein can be an effective diagnostic marker for detection and classification of TGCTs. To explore this possibility, we had examined TSPY expression in 61 cases of seminomas (42, 9 and 10 cases of stage I, II and III respectively) and 70 cases of nonseminomas (embryonal carcinoma, yolk sac tumor, and teratoma at different clinical stages). Of these, 27 cases of seminomas and 26 cases of nonseminomas harbored CIS/ITGCNU adjacent to the respective tumor sites (Table 2). They were analyzed independently from the respective tumor sites within the specimens. Eight cases of nonseminomas contained seminomatous components, which were excluded in the analysis. Immunostaining was performed by a technical staff and analyzed independently by an attending pathologist. TSPY staining intensity was graded from 0 to 3 corresponding to none to strong respectively while the extent of staining was graded from 0 to 3 representing no to >60% of tumor cells positive (Table 3). The overall TSPY grading was calculated as the sum of the two grades. Hence, this grading system indicates that a 0 score means no cells were positive for TSPY while a 6 score means that >60% of cells are strongly stained with TSPY antibody. Intermediate scores suggest that staining was either heterogeneous or variation in intensity on the tumor cells. Our results showed that CIS/ITGCNU showed the most intense and comprehensive staining of the tumor cells (Figure 4), irrespective of their origins from either seminoma or nonseminoma specimens. In fact, almost every CIS/ITGCNU cell was positive for TSPY in most specimens. All, but 10 cases, seminomas showed significant and intense staining with the TSPY antibody. The percentages of TSPY negative samples seemed to be higher in the late clinical stage III than the earlier stages. Nonseminomas, excluding the CIS/ITGCNU or seminoma components present within the specimens, showed only background staining for TSPY. No specific staining pattern could be identified among these minimally stained samples. All samples that were negative for TSPY by immunohistochemistry were also negative in Western blot analysis using the same TSPY antibody. The present results clearly support the notion that TSPY is a significant diagnostic marker for CIS/ITGCNU, the precursor, and seminomas, and a key differential marker for classification of TGCTs.

### 3.5 TSPY Up-regulates Chromosome 12p13 Genes

Numerous studies suggest that genes on chromosome 12 band p13 could play important functions in the pathogenesis of TGCTs [21–24]. To explore the correlation between the expression levels of TSPY and selected 12p genes, we have analyzed available microarray data of normal testis, clinical TGCT and CIS/ITGCNU samples [26,27] using hierarchical clustering analysis. The results showed that TSPY expression patterns in clinical and normal samples detected by microarray analyses (Figure 5A–B, horizontal blue boxes) were closely related to those demonstrated by immunostaining, Western blotting and RT-PCR studies (i.e. Figure 1–4). TSPY was expressed at moderate levels in normal testes (Figure 5A, NT1-3) and at elevated levels in CIS/ITGCNU (Figure 5A, ITGCNU1-3), most seminoma (Figure 5A, SE1 and 5B,

SE5-7) and spermatocytic seminoma samples (Figure 5B, SS1-5), but reduced levels in most embryonal carcinomas (Figure 5A, EC1 and 5B, EC2, EC4-6) and 3 seminomas (Figure 5A–B, SE2-4). Dysgerminomas also showed minimal (background) levels of TSPY hybridization (Figure 5B, DG1-3). Since dysgerminomas are female ovarian seminomatous germ cell tumors lacking the Y chromosome, it is expected that no TSPY should be expressed in them. It is interesting to note that some testicular seminomas, such as SE2-3 (Figure 5A) and SE4 (Figure 5B) showed reduced levels while some nonseminomas, such as EC3 (Figure 5B), showed elevated levels of TSPY expression. Such heterogeneity in TSPY expression was also observed in immunostaining studies (Table 2 and Figure 4). Hence, TSPY expression patterns detected by microarray analyses of clinical samples follow similarly those observed by immunological techniques.

Previously we demonstrated that ectopic expression of TSPY in cultured HeLa cells up-regulated various oncogenes and pro-growth genes, including CCND2, and repressed apoptotic genes and growth inhibitors [25]. A re-examination of our microarray data showed that selected 12p genes, including the CCND2 (cyclin D2), KRAS (v-Ki-ras2 oncogene), NANOG (a homeobox stem cell factor), FOXM1 (the forkhead box M1), WNK1 (WNK lysine deficient protein kinase 1) and CD9 (a member of the transmembrane 4 superfamily), were also up-regulated either directly or indirectly in HeLa cells by the ectopic expression of TSPY (Figure 5C). To confirm such up-regulation, specific primer pairs corresponding to the respective transcripts of these genes (Table 1) were used in a semi-quantitative RT-PCR analysis of RNAs derived from HeLa cells harboring/expressing TSPY (Figure 5D, +) and those containing the vector alone (Figure 5D, −). Our results confirmed the initial microarray analysis of these cell populations (Figure 5D) and showed higher levels of transcripts of these 12p genes in HeLa cells expressing TSPY than cells harboring the vector alone. Interestingly, the expression levels of these genes, estimated by semi-quantitative RT-PCR were higher than those estimated by our initial microarray analysis, as previously observed [25]. We surmise that these differences could be attributed to the respective sensitivity of the two methods.

Expression of the selected 12p genes was low in normal testes (Figure 5A, NT1-3), increased in CIS/ITGCNU (Figure 5A, ITGCNU1-3) and further in seminomas (Figure 5A–B, SE1, SE4-7), and was at highest levels in the nonseminomatous embryonal carcinomas (Figure 5A–B, EC1–6). The gradual increases in the expression of 12p genes could be associated with the progression of the tumorigenic process(es) of TGCTs. Significantly, the expression patterns of TSPY and selected 12p genes in CIS/ITGCNU and most seminoma samples (Figure 5A–B, vertical yellow boxes) resembled closely with that of the HeLa cells ectopically expressing TSPY (Figure 5C), suggesting a possible link between elevated TSPY expression and up-regulation of the selected 12p genes in these types of germ cell tumors. Notably, further increases of 12p gene expression in some seminomas (i.e. SE2-4) and most embryonal carcinomas were associated with reduced TSPY expression in these samples. Since gain of chromosome 12p and/or amplification of certain portion of this chromosome were associated with the development of advanced or invasive germ cell tumors, such differential expression of TSPY suggests that it might play a significant but temporal role in the evolution of the tumorigenic germ cell genome.

## 4. Discussion

The present study demonstrated the significant association of TSPY expression with TGCT precursor, CIS/ITGCNU, and majority of testicular seminomas. Examination of available expression microarray data confirmed such preferential expression pattern of this Y chromosome gene in different types of TGCTs. TSPY is co-expressed with other established germ cell tumor markers, i.e. PLAP, c-KIT, OCT4 and the proliferative marker, Ki-67, in a majority of tumor cells [19,32,34], thereby confirming its significant as a diagnostic marker

for CIS/ITGCNU and seminomatous tumors. By establishing a crude grading system, we were able to efficiently demonstrate such differential TSPY expression and to distinguish seminomas from nonseminomas. Hence, TSPY is an important diagnostic marker for the detection and/or classification of the various subtypes of TGCTs for routine pathological and clinical analysis.

Gonadoblastoma is a rare germ cell tumor occurring most frequently in XY sex-reversed and intersex patients who harbor residual Y chromosome materials [4–6,8–10]. Gonadoblastoma and TGCT precursor, CIS/ITGCNU, share significant similarities in their morphology and tumor behavior [24,35,36]. The abundant expression of TSPY in both gonadoblastoma and CIS/ITGCNU further supports such postulation of common origin(s) of these two precursors for aggressive germ cell tumors [15,36]. The identification of TSPY as a significant candidate for GBY, thereby, associates this repeated Y chromosome gene with the pathogenesis of the more common TGCTs among young men. Since CIS/ITGCNU has been considered to be the precursor for both seminomas and nonseminomas, i.e. embryonal carcinoma, teratoma and yolk sac tumor [15,37–39], the differential expression of TSPY between seminomas and nonseminomas implies that these two types of germ cell tumors have taken on separate differentiation pathways in their tumorigenic processes. These findings suggest that testicular seminomas evolve along the germ cell lineage while nonseminomas could be activated to more pluripotent states capable of addition differentiation into other cell types [19,24]. Potentially, these adult pluripotent cells could possibly used as somatic stem cells for transdifferentiation studies, including those for gonocytes [17].

Several studies have demonstrated the expression of TSPY in gonadoblastomas, testicular seminomas, intracranial germ cell tumors, prostate cancer, hepatocellular carcinoma and melanoma of male origins [13,14,16,40–42]. Previously, we demonstrated a preferential expression of a variety of alternatively spliced TSPY transcripts coding for different abbreviated isoforms of TSPY protein in prostate cancer samples [16]. Interestingly, similar enrichment of TSPY isoforms at both RNA and protein levels in seminoma samples were also observed in the present study, suggesting the same preferential expression of the abbreviated forms of this Y-encoded protein in this type of TGCTs. Currently it is uncertain if these polymorphic TSPY proteins serve the same or different biological function(s). It will be interesting to determine if tumors from other tissues, e.g. intracranial, hepatocellular carcinoma and melanoma, also preferentially express such abbreviated forms of the TSPY protein.

TSPY is expressed in early gonocytes in prenatal and postnatal testes [32] and spermatogonia and, to a certain extent, round spermatids of adult testis [28]. It has been postulated to serve a certain role(s) in stem germ cell proliferation and/or male meiosis [4,7]. In particular, disruption/delay of fetal germ cell development could play significant role in the pathogenesis of TGCTs [37,43–45]. Hence, TSPY expression in normal fetal gonocytes/prespermatogonia and adult spermatogonial stem cells affirms its possible functions in male stem germ cell differentiation while its ectopic expression in the TGCT precursor, CIS/ITGCNU, and seminomas supports the possibility of a delayed or a reactivated fetal programming in these tumor germ cells. Currently the exact mechanisms of TSPY action(s) at the molecular and cellular levels are uncertain, its expression in germ cell tumors and cancers of somatic origins suggests that it might exert a proliferative function(s) at the cellular level when it is ectopically expressed in these cells. Indeed, recently we had showed that over-expression of TSPY in cultured somatic cells potentiated cell proliferation in vitro and tumor formation in nude mice [25]. Cells over-expressing TSPY transited the $G_2$/M phase more rapidly than those without such expression. Transcriptome analysis demonstrated that pro-growth genes and selected oncogenes were up regulated while apoptotic factors and cell cycle inhibitors were down regulated in these TSPY expressing cells. Ontology analysis of the differentially expressed genes suggested that pathways involved in cell cycle regulation were mostly affected.

A more focused analysis in the present study demonstrated that ectopic expression of TSPY in HeLa cells up-regulated selected chromosome 12p genes, postulated to be involved in the evolution of TGCTs [21,24]. Currently the exact mechanism(s) by which TSPY alters the expression these chromosome 12p genes is unknown. TSPY has not been demonstrated to be a transcription factor, and hence, it could likely influence such changes in gene expression in an indirect manner(s). Further, it is interesting to observe that GAPDH is also located at chromosome 12p13, it was not up-regulated in HeLa cells over-expressing the TSPY transgene (Figure 5D). Hence, TSPY effects could be selectively on genes residing on this chromosome region. Significantly, among these 12p genes up-regulated by ectopic expression of TSPY, both CCND2 and FOXM1 are key players in cell cycle regulation. CCND2 is the gene for cyclin D2 that binds to and activates the cyclin dependent kinase, CDK4/6, essential for the cell to exit $G_0$ and enter $G_1$ or to cycle from $G_1$ to S phase [46,47]. By up-regulating cyclin D2, TSPY could have a positive effect(s) on cell proliferation and tumor initiation. Significantly, FOXM1 encodes a key transcription factor directly binding to the promoters and regulating the transcription of many genes coding for various cell cycle regulators, including cyclin B1, Polo-like kinase (PLK1), CENP-F, Cdc25B phosphatase, and Auro B kinase, important for $G_2$/M stage [48–50]. The up-regulation of FOXM1 by ectopic TSPY expression might be responsible for the rapid transition of the cells through $G_2$/M, previously demonstrated [25]. More importantly, an expedited progression through this stage of the cell cycle could affect various $G_2$ and mitotic checkpoints, essential for DNA repair and orderly cell division [51,52]. Inactivation of such checkpoints will likely enhance chromosome nondisjunction and/or genomic instability, thereby increasing mutational events and exacerbating the tumorigenic process(es) [53]. We surmise that such TSPY effects could be important for the pre-malignant precursor, i.e. CIS/ITGCNU, to enter the cell cycle and for seminomatous cells to maintain their oncogenic properties. The mutational pressure exerted by ectopic TSPY expression could result in either a gain of chromosome 12p or amplification of certain genes therein that favors tumorigenic progression. Indeed, gain/amplification of chromosome 12p seems to be related to acquisition of pluripotency or “stemness” properties by the tumor germ cells [20,54,55]. The development of aggressive/pluripotent phenotypes, thereby, could minimize the necessity for TSPY functions, resulting in reduction in its expression in late stages of TGCTs.

Currently, we are uncertain what role TSPY might play in spermatocytic seminoma. This type of tumors is postulated to derive from primary spermatocytes and gain of chromosome 9 is the only consistent genomic anomaly [27]. In fact, 12p genes are not up-regulated in this type of germ cell tumors (Figure 5B). TSPY could potentially affect other genes residing elsewhere in the human genome that favor the oncogenic process(es) for this type of seminomas. Nevertheless, TSPY dysregulation of the cell cycle, in combination with other oncogenic events, including activation of proliferation and stem cell genes as well as expression of specific cell cycle controlling microRNAs [56], could play critical roles in the pathogenesis and/or progression of gonadoblastoma and TGCTs.

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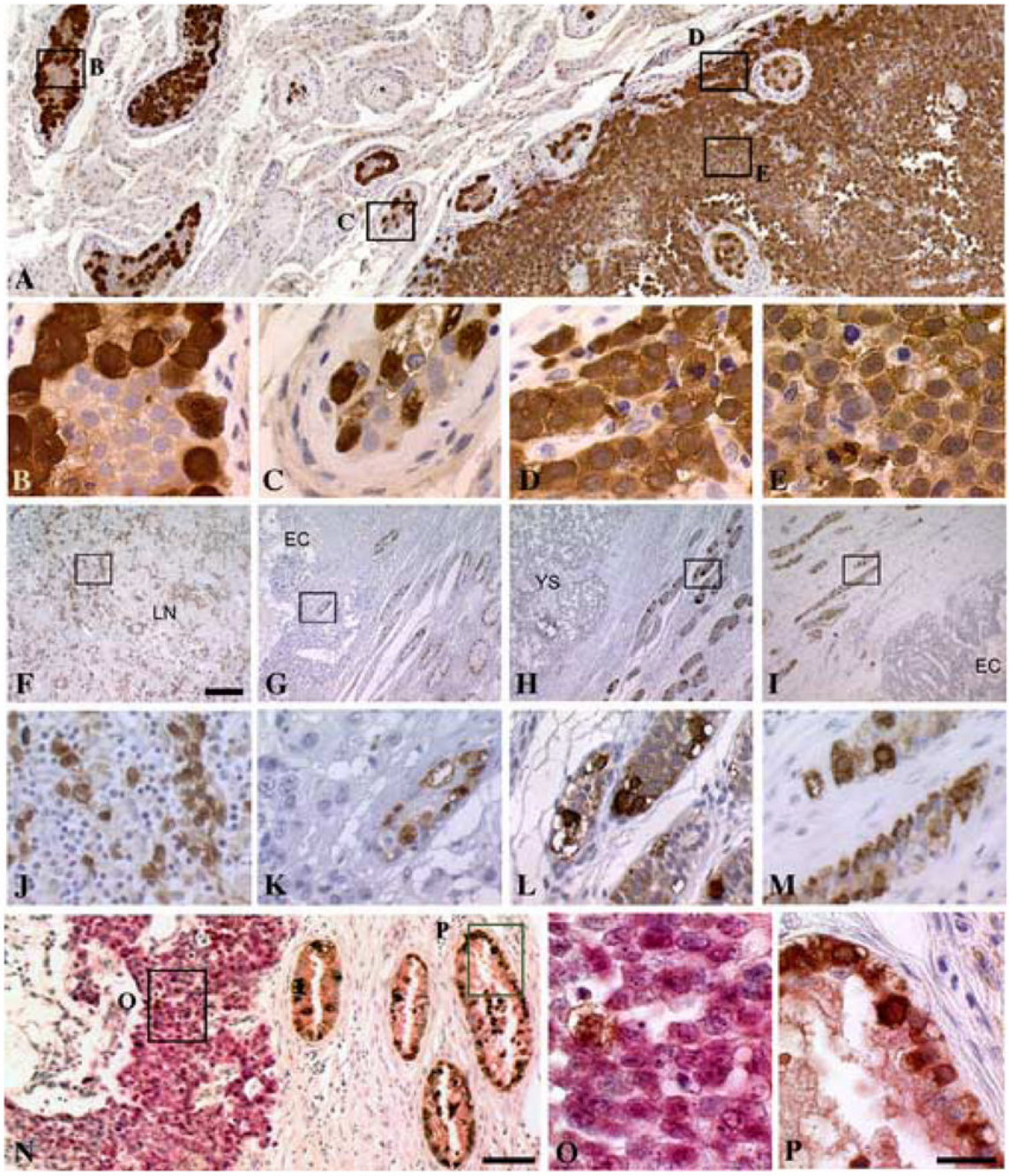


**Figure 1.**

Preferential TSPY expression in testicular seminoma and its precursor, CIS/ITGCNU. A) An example of intense TSPY immunostaining of seminoma (right) harboring adjacent CIS/ITGCNU cells (left) in a 34-year old patient. B–E) Enlarged views of boxed areas in A showing CIS/ITGCNU (B, C) and seminoma (D, E) components. Immunostaining of TSPY on F) a case of metastatic seminoma in lymph node, and G–I) selected nonseminomas harboring adjacent CIS/ITGCNU cells. TSPY was intensely positive for metastatic seminoma cells (J), and CIS/ITGCNU cells but not the respective nonseminomas (K–M). N) Double immunostaining of TSPY and OCT4 on an embryonal carcinoma (left) with adjacent CIS/ITGCNU (right) of a 35-year old patient. O–P) Enlarged views of corresponding boxed areas in N. LN = lymph

node; YS = yolk sac tumor; EC = embryonal carcinoma. Bar in F represents 400 μm in F–I; bar in N represents 100 μm in A, J–N; and bar in P represents 20 μm in B–E, O–P respectively.

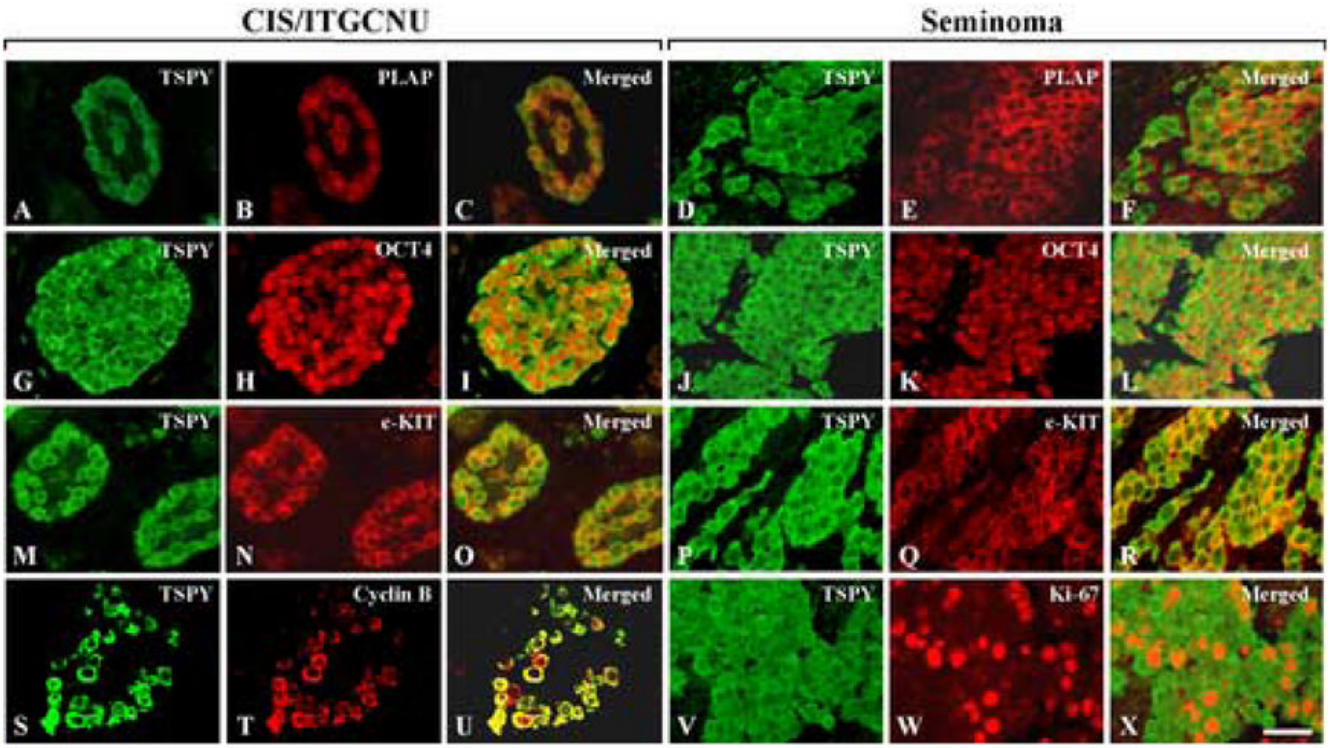


**Figure 2.**

Co-expression of TSPY and various tumor markers in CIS/ITGCNU (left 3 columns) and seminoma (right 3 columns) tumor germ cells. Double immunofluorescence of TSPY (green in A, D, G, J, M, P) and PLAP (red in B, E), OCT4 (red in H, K), and c-KIT (red in N, Q) showed that these tumor markers were expressed in the same tumor germ cells, as indicated in merged images (yellow-orange in C, F, I, L, O and R). TSPY (green in S and V) was co-expressed with cyclin B1 (a potential interactive partner, red in T) and the Ki-67 proliferative marker (red in W) in the same tumor germ cells, as revealed in respective merged images (yellow-orange in U and X). These specimens were derived from patients with ages between 27 to 56 years old. Bar in X represents 20 μm in all images.

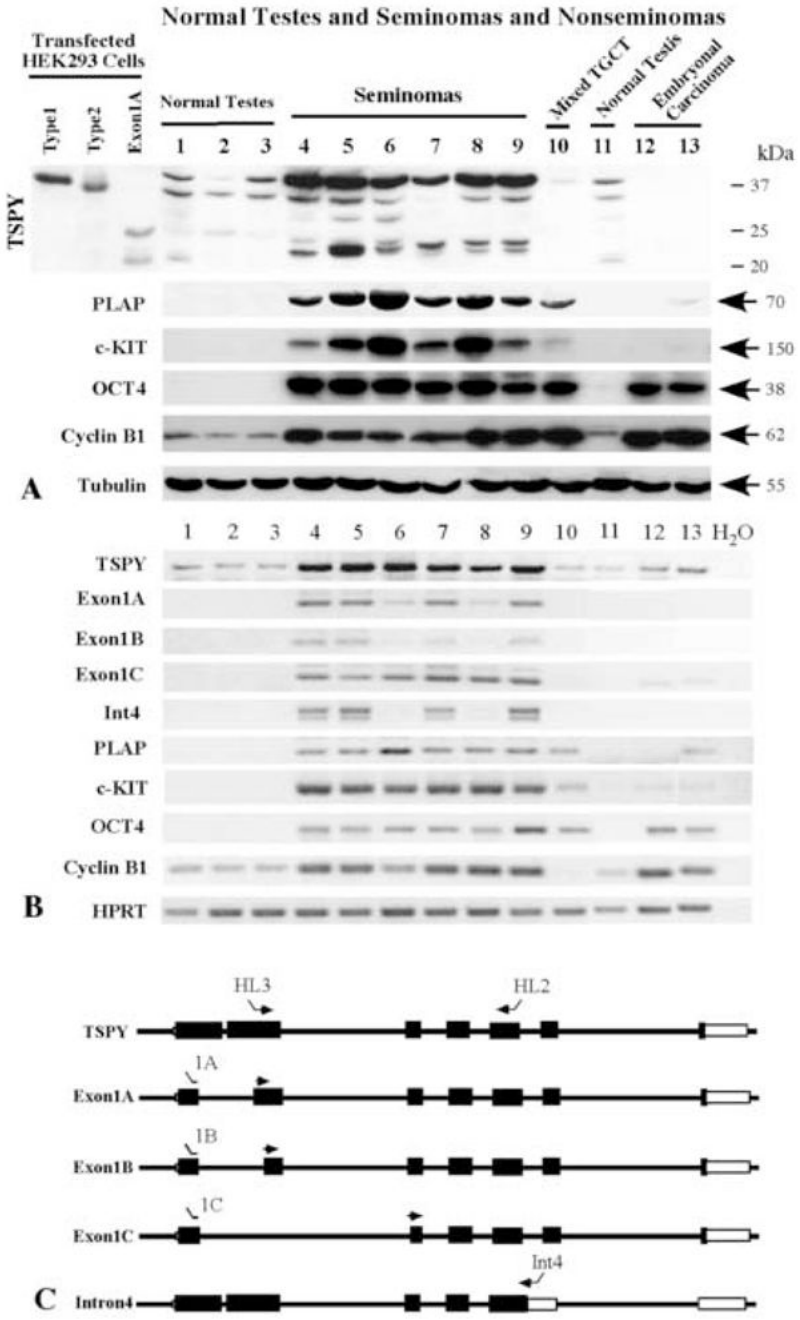


**Figure 3.**

Protein and RNA analyses of gene expression in normal testes, seminoma and nonseminoma specimens. A) Western blot analyses of various samples with a TSPY antibody showed multiple reactive protein bands, representing different isoforms of TSPY in protein lysates of normal testes (lanes 1–3, 11), seminomas (lanes 4–9) and nonseminomas (lane 10, 12, 13). Some of these TSPY bands corresponded to those from HEK293 cells transfected with DNA plasmids expressing various TSPY isoforms (left 3 lanes, type 1 and 2 = full length cDNA, Exon1A = cDNA of alternatively spliced transcript). The amounts of TSPY proteins were the highest among the seminoma specimens, low but detectable levels in normal testes and minimal amounts or none among the nonseminomas. Re-probing of the same or parallel filters showed

similar expression patterns for PLAP, c-KIT and, to a certain extent, OCT4 and cyclin B1. The latter markers also expressed at high levels in selected nonseminoma specimens (e.g. mixed germ cell tumor in lane 10 and embryonal carcinomas in lanes 12 and 13). The relative amounts of tubulin seemed to be quite even among all samples. B) RT-PCR analyses of transcripts for TSPY isoforms (TSPY=total transcripts; Exon1A, Exon1B, Exon1C and Int4 = alternatively spliced transcripts), and germ cell tumor markers (PLAP, c-KIT, OCT4, and cyclin B1) in the same samples used in Western blotting in A. Successful amplification of RT-PCR products with primer sets specific for the transcripts of the respective isoforms suggested that these variant TSPYs were present in the corresponding samples, particularly the seminomas (lanes 4–9). Although semi-quantitative in nature, the amounts of the RT-PCR products paralleled the intensities of Western blot signals (detected in A) while the same analysis of a reference gene (HPRT) showed relatively even amounts of RT-PCR products among all samples. All RT-PCR images were obtained from negative printing of the respective ethidium bromide staining of the agarose gels. C) Diagrammatic illustration of major variant transcripts originated from the expression of the TSPY tandem arrays on the human Y chromosome. Arrows indicate the positions of respective primers in the structural gene used to detect these variant transcripts by RT-PCR analysis (B). Solid and open boxes represent coding and non-coding sequences of the variant transcripts.

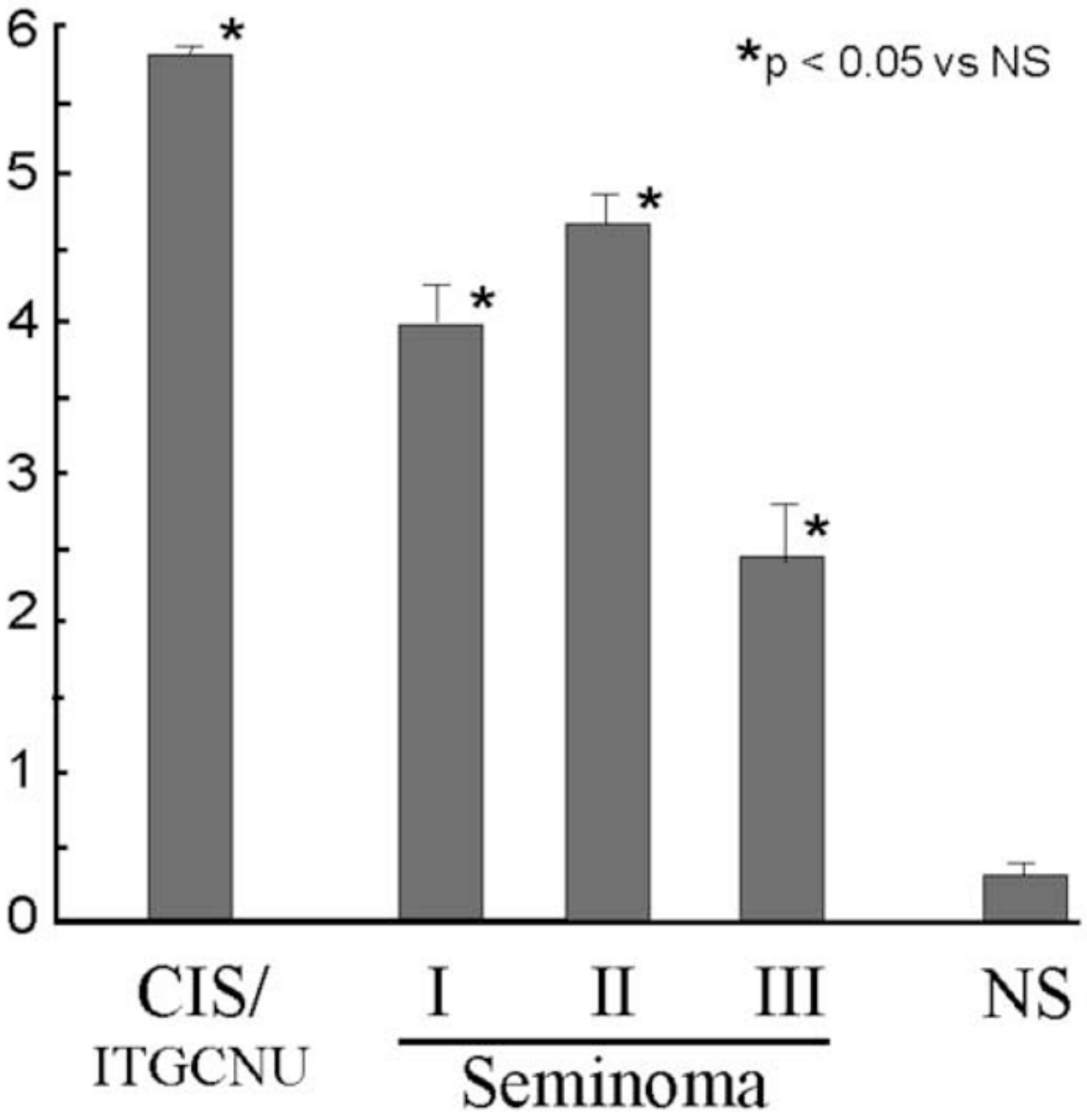


**Figure 4.**

Relative TSPY immunostaining of tumor cells in various subtypes of TGCTs, based on a crude grading system (as described in Table 3). The CIS/ITGCNU, precursor for all TGCTs, showed the most intense and extensive staining while seminomas at clinical stages I, II and III were positive but less intense than the CIS/ITGCNU. Nonseminomas at all clinical stages showed minimal staining with the TSPY antibody under the same conditions, suggesting that they did not express TSPY to any significant levels. TSPY seemed to express at higher levels in early germ cell tumors, but declined towards more advanced clinical stages among the CIS/ITGCNU and seminoma samples. Such differential TSPY expression suggests that TSPY could be an efficient marker for identification of pre-malignant precursors (CIS/ITGCNU) and

classification of various subtypes of TGCTs. Student's t-test showed that the p value of these samples were <0.05 when compared with nonseminomas (NS), suggesting that they were significantly different from the NS samples in TSPY immunostaining.

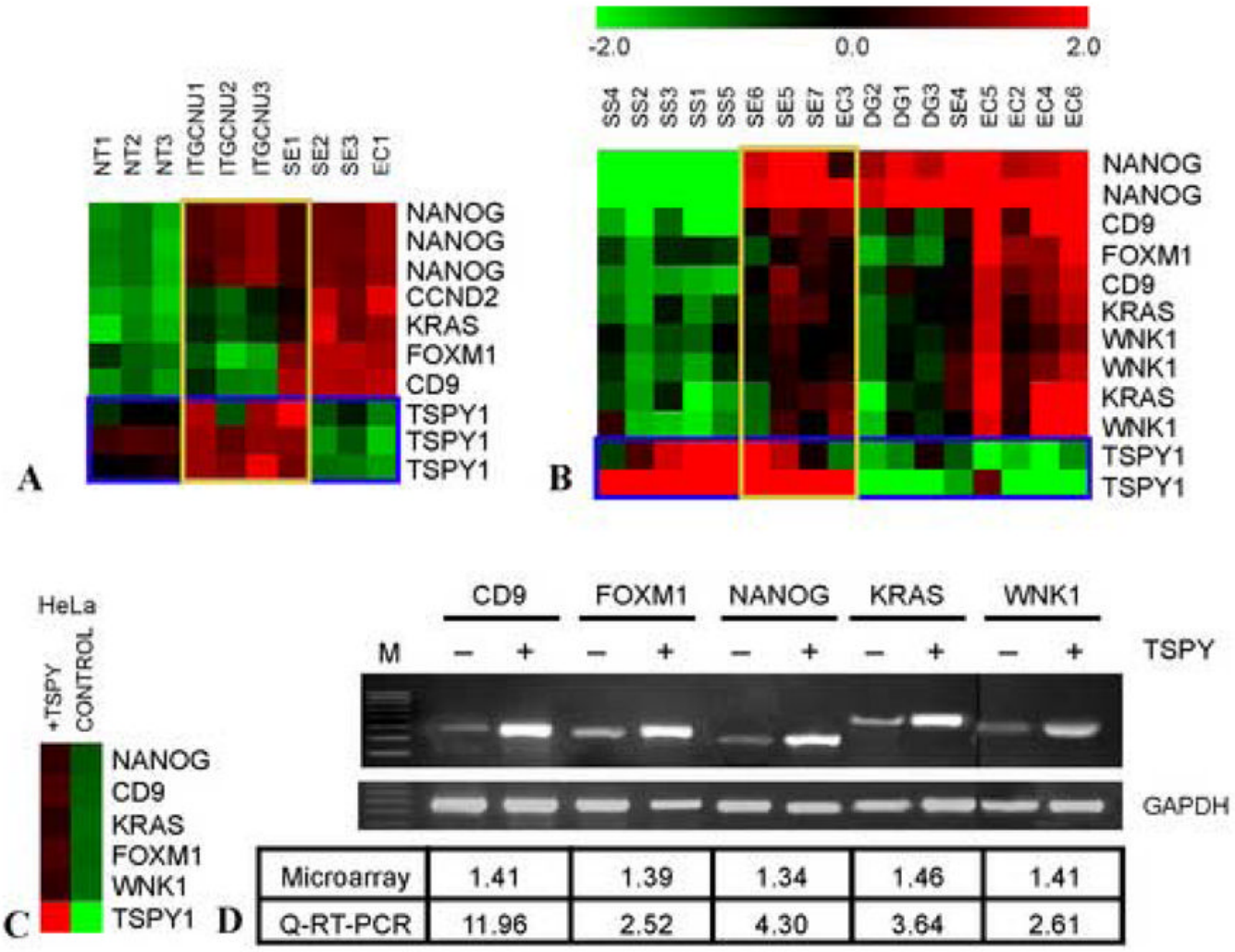


**Figure 5.**

TSPY expression is associated with up-regulation of selected chromosome 12p genes, involved in TGCTs. A–B) Hierarchical clustering analysis of microarray data on TSPY and selected 12p13 genes from GEO datasets (A, see Methods) and published data (B, Looijenga, 2006). Gene candidates among the list may occur more than once due to duplicate probes, indicating the consistent trend of gene expression in the group. The expression patterns of CIS/ITGCNU and seminoma samples (vertical boxed regions in A and B) resemble closely that for HeLa cells ectopically expressing TSPY transgene (C). C–D) Ectopic TSPY expression up-regulates 12p genes. C) Hierarchical clustering analysis of selected 12p genes in HeLa cells over-expressing TSPY (HeLa) and control cells (control). D) Semi-quantitative RT-PCR analysis of RNAs derived from HeLa cells expressing (+) and lacking (−) TSPY, using specific primers for 12p genes. GAPDH was used as a control. The relative ratios of TSPY expression were calculated as signals from HeLa+TSPY/HeLa+control, as from microarray and Q-RT-PCR analyses (lower panel).

**Table 1**

Primer sets used in RT-PCR analysis of TSPY isoforms, germ cell tumor marker and reference genes

| Primers | Sequence |
|---|---|
| TSPY-HL2 | 5′-GTCTGCGGCGATAGGCCTCCACTT-3′ |
| TSPY-HL3 | 5′-TCGGCAGCGGGAAAAGATGGAGCG-3′ |
| TSPY-Exon1A | 5′-GCACAGGCCTTGGTGGAGCTGGAG-3′ |
| TSPY-Exon1B | 5′-GCACAGGCCTTGCGGGAAAAGATGG-3′ |
| TSPY-Exon1C | 5′-GCACAGGCCTTGATGTCAGCCCTG-3′ |
| TSPY-Intron4 | 5′-CGGGAAAGGCCTCATCAGGGCTC-3′ |
| PLAP-F | 5′-CAACTTCCAGACCATTGGCTTG-3′ |
| PLAP-R | 5′-TTACCACTCCCACTGACTTCCCTG-3′ |
| KIT-F | 5′-GGACTTGAGGTTTATTCCTGACCC-3′ |
| KIT-R | 5′-GCTTGCTTTGGACACAGACACAAC-3′ |
| OCT4-F | 5′-TGGGGGTTCTATTTGGGAAGG-3′ |
| OCT4-R | 5′-GTTCGCTTTCTCTTTCGGGC-3′ |
| Cyclin B1-F | 5′-TGGGGACATTGGTAACAAAGTCAG-3′ |
| Cyclin B1-R | 5′-TGGGCTTGGAGAGGCAGTATCAAC-3′ |
| HPRT-F | 5′-CCTGCTGGATTACATTAAAGCACTG-3′ |
| HPRT-R | 5′-GTCAAGGGCATATCCAACAACAAAC-3′ |
| CD9-F | 5′-GGATATTCCCACAAGGATGAGGT-3′ |
| CD9-R | 5′-GATGGCTTTCAGCGTTTCCC-3′ |
| FOXM1-F | 5′-TTGCCCGAGCACTTGGAATC-3′ |
| FOXM1-R | 5′-GGGGAGTTCGGTTTTGATGGT-3′ |
| NANOG-F | 5′-TGATTTGTGGGCCTGAAGAAAA-3′ |
| NANOG-R | 5′-GAGGCATCTCAGCAGAAGACA-3′ |
| KRAS-F | 5′-GGACTGGGGAGGGCTTTCT-3′ |
| KRAS-R | 5′-GCCTGTTTTGTGTCTACTGTTCT-3′ |
| WNK1-F | 5′-ACCCTCGGTTGTTCCAGTC-3′ |
| WNK1-R | 5′-ACACATGAGGAGTTGATATGGGA-3′ |
| GAPDH-F | 5′-ATGGGGAAGGTGAAGGTCG-3′ |
| GAPDH-R | 5′-GGGGTCATTGATGGCAACAATA-3′ |

**Table 2**

TSPY staining of TGCTs

| TGCTs | Stage | Cases | +TSPY | +CIS/ITGCNU |
|---|---|---|---|---|
| Seminoma | I | 42 | 37 | 18 |
| | II | 9 | 9 | 4 |
| | III | 10 | 5 | 5 |
| Non-seminoma* | I | 34 | 4 | 18 |
| | II | 8 | 0 | 5 |
| | III | 28 | 6 | 13 |

* Nonseminoma specimens consisted of embryonal carcinoma, yolk sac tumor, teratoma, and mixed germ cell tumors with seminoma components (which, together with CIS/ITGCNU, were excluded in TSPY immunostaining scoring).

**Table 3**

Scoring System for TSPY Staining of TGCTs

| Staining Intensity | SI |
|---|---|
| No staining | 0 |
| Weak | 1 |
| Intermediate | 2 |
| Strong | 3 |
| Positive cells | PC |
| None | 0 |
| <30% | 1 |
| 30–60% | 2 |
| >60% | 3 |
| Overall Score = SI + PC | |