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Correlation between retinoblastoma gene expression and differentiation in human testicular tumors

(testicular neoplasms/tumor suppressor genes/tumor cell differentiation/oncogenes)

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ABSTRACT **Inactivation of the retinoblastoma gene (*RB* gene) is associated with the development of several human malignancies including retinoblastomas, some osteo- and soft tissue sarcomas, small cell lung cancer, and possibly breast and bladder cancers. To our knowledge, this gene has not been evaluated in human germ-cell malignancies. In this study 67 primary testicular germ-cell tumors and 4 testicular non-germ-cell malignancies were examined to determine the prevalence and nature of *RB* gene alterations. Decreased expression of *RB* gene mRNA was found in all testicular germ-cell tumors (both seminomas and nonseminomas) examined. The RB protein could not be detected by immunohistochemical analysis in the undifferentiated cells of any germ-cell tumors whereas the differentiated malignant cells present in 14/15 teratocarcinomas expressed the protein. No gross alterations of the *RB* gene were found at DNA level in any of the examined specimens. This and the presence of the RB protein in the more differentiated tumor cells of teratocarcinomas suggest that changes in transcript levels rather than mutation(s) of the gene may be responsible for the absent or decreased *RB* expression in human germ-cell tumors. To date studies on the mechanism of *RB* regulation have demonstrated that it occurs at the protein level by phosphorylation of the p105 gene product. The findings presented here indicate that additional regulation might occur at the transcript level.**

Testicular tumors represent the most frequent solid tumor of males 20–40 years old. In the United States, the age-adjusted annual incidence rate is 3–4/100,000 (1). Ninety percent of testicular tumors originate from germ-cell tissue and 10% are derived from interstitial tissue. The World Health Organization classifies testicular germ-cell tumors (GCTs) into seminomas and nonseminomas. Nonseminomas are further subdivided into various subgroups including embryonal carcinomas, choriocarcinomas, as well as teratocarcinomas and other mixed tumors made up of more than one histologic type. Teratocarcinomas represent a relatively unique entity among human tumors in that these malignancies contain undifferentiated tumor cells (embryonal carcinoma component) and differentiated tumor cells forming a wide variety of tissue structures such as cartilage, epithelia, bone, muscle, etc. (teratoma component) (2). Data indicate that both seminomas and nonseminomas originate from the same precursor cell, the intratubular germ-cell neoplasia, originally termed the "carcinoma-*in-situ*" (CIS) cell (3–5). The events involved in initiation and progression of testicular GCTs are unknown; however, there are a number of cytogenetic studies indicating that these tumors frequently contain gross genetic alterations (6–14). To date, a small number of studies evaluating molecular genetic abnormalities in GCTs have been published, and all of these deal with alterations in protooncogenes (15–20). In this study, human testicular cancers were evaluated to determine whether alterations of a tumor suppressor gene, the retinoblastoma gene (*RB* gene), or its expression occur in these malignancies.

The *RB* gene is a tumor suppressor gene that was identified by virtue of its alteration in childhood retinoblastoma. This malignancy is an ocular tumor that develops from immature retinoblasts usually before 4 years of age (21–23). Identification and cloning of this gene have allowed for molecular genetic studies in human tumors. These studies have shown that the gene is partially or completely deleted in 15–40% of primary retinoblastomas and that gene expression is altered in the majority of retinoblastomas (24–26). In addition, inactivation of the *RB* gene has been demonstrated in a number of other human tumors including osteosarcomas and soft tissue sarcomas (25, 27–31), small cell lung cancer (SCLC) (32), and possibly, breast (33, 34) and bladder malignancies (35). The *RB* gene encodes a 105-kDa protein, believed to function as a cell cycle regulator (36–43), that is constitutively expressed in all normal human and rodent tissues studied thus far (24, 44). Recent studies report that transfection of the gene into tumor cells from which it was deleted reverses the malignant phenotype. These data indicate that *RB* deletions may play a role in the pathogenesis of those cancers in which they occur rather than being an epiphenomenon (45, 46).

MATERIALS AND METHODS

Fresh primary testicular tumor tissue was obtained at initial surgery and stored in liquid nitrogen or at −70°C. Adjacent normal (tumor-free) tissue was also obtained whenever possible. Tumor tissue was collected from 67 patients presenting with testicular lesions that were ultimately diagnosed as GCTs. In addition, adjacent nonmalignant tissues from 58 of these specimens were obtained. Other testicular tissue specimens in this cohort included four non-germ-cell testicular tumors (two Leydig cell tumors, one metastasis from a SCLC, and one non-Hodgkin lymphoma) and six nonmalignant testes (three partially necrotic testes after recurrent incomplete torsions, two normal testes from orchiectomy of prostatic carcinoma patients, and one chronic orchitis).

Northern Blot Analysis. RNA was extracted from these specimens with guanidinium thiocyanate, purified, analyzed, and processed as described (47). Two DNA fragments of the

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Abbreviations: GCT, germ-cell tumor; SCLC, small cell lung cancer; CIS cell; carcinoma-*in-situ* cell.
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RB gene [a PCR-generated 0.9-kilobase (kb) fragment representing the 5′ portion of the gene (pR65) and a 3.8-kb cDNA fragment representing the 3′ portion of the gene] was the generous gift of R. Takahashi (Center for Biotechnology, Baylor College of Medicine, The Woodlands, TX). The 3′ fragment was radiolabeled with [^{32}P]dCTP by random priming (48) to a specific activity of 1×10^9 cpm/μg (31). Northern blots were hybridized to the 3′ probe of the *RB* gene in 50% (vol/vol) formamide/1 M NaCl/0.4% SDS/herring sperm DNA (400 μg/ml)/10% (wt/vol) dextran sulfate at 42°C for 24 hr. Filters were washed in 2× standard saline citrate (SSC)/1% SDS at room temperature and in 2× SSC/1% SDS at 65°C, subsequently stripped, and rehybridized to a human β-actin probe (603-base-pair *Bam*HI–*Eco*RI fragment) under identical hybridization and washing conditions. Autoradiograms were scanned and relative *RB* expression levels were compared by densitometry. Tumors were grouped into two categories of RB mRNA expression: 1.5- to 2-fold decrease or 3- to 15-fold decrease.

Immunohistochemistry. Acetone-fixed frozen sections of each specimen were stained with hematoxylin/eosin and examined microscopically to confirm the presence of tumor cells. Immunohistochemistry was performed on 5-μm formalin-fixed frozen sections using the Rb-PMG3-245 antibody (Phar Mingen) and the avidin–biotin complex (ABC) technique (28). Serial sections were treated with Mel-5 (Signet Laboratories, Dedham, MA), an antibody to human melanosomes, as a negative control. All sections were counterstained with methylene blue to verify presence of intact nuclei.

Southern Blot Analysis. DNA was extracted with guanidinium thiocyanate, purified, analyzed, and processed as described (47). *Hin*dIII digestion was performed. Both fragments of the RB probe were radiolabeled with [^{32}P]dCTP by random priming (48) to a specific activity of 1×10^9 cpm/μg (31). Probe hybridization was sequentially done with the 3′ fragment and the 5′ fragment of the RB probe at 65°C for 24 hr using 5× SSPE/5× Denhardt's solution/0.5% SDS/herring sperm DNA (100 μg/ml) as hybridization solution (1× SSPE = 0.18 M NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA). To assure equal loading, a probe derived from sequences on chromosome 13 unrelated to *RB*, termed p9D11, was simultaneously hybridized (31, 49). Membranes were washed in 2× SSC at room temperature, in 0.1× SSC/0.1% SDS at room temperature, and in 0.1× SSC/0.1% SDS at 50°C, consecutively. Additionally, multiple cuts using *Msp* I, *Taq* I, *Apa* I, and *Dra* I as restriction enzymes were performed. These blots were then hybridized with chromosome 13 polymorphic probes: 7F12, ESD, 9D11, 1E8, 9A7, and 7D2.

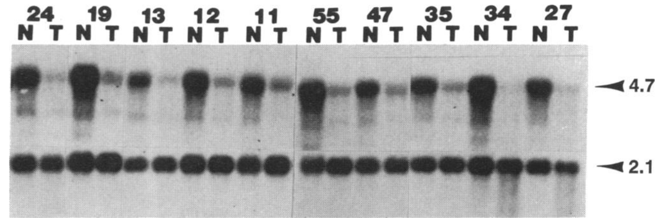

FIG. 1. Northern blot analysis of the *RB* gene RNA. Distinct reduction of the RB mRNA (4.7 kb) in the tissues of malignant GCTs of various histologies (lanes T) and expression in macroscopically normal adjacent testicular tissues (lanes N). The 2.1-kb transcript is β-actin. Numbers above lanes are specimen identification numbers.

RESULTS

Expression of the *RB* gene was studied at the RNA level by Northern blot analyses in 40 GCTs and 21 macroscopically normal adjacent testicular tissue samples. A 3- to 15-fold decrease in expression of the RB transcript was found in 37/40 GCTs compared to levels expressed in the adjacent normal testicular tissue (Fig. 1). The levels observed in the adjacent normal testes are consistent with the constitutive levels reported in other normal tissues (24, 44) and those found in 2 normal testicular specimens and 4 nonmalignant testicular lesions studied in this cohort (data not shown). In the remaining three GCTs, the transcript was decreased to a lesser extent (1.5- to 2-fold reduction) compared to levels found in adjacent normal tissue. Conversely, normal transcript levels were detected in the Leydig cell tumors and the testicular lymphoma (data not shown). RB mRNA, however, was absent in the testicular metastasis from the SCLC. This observation is consistent with data on RB expression in SCLC (32). No transcript of altered size was detected in any of the testicular tissue specimens examined.

RB protein expression was analyzed immunohistochemically using a monoclonal antibody (Rb-PMG3-245) that rec-

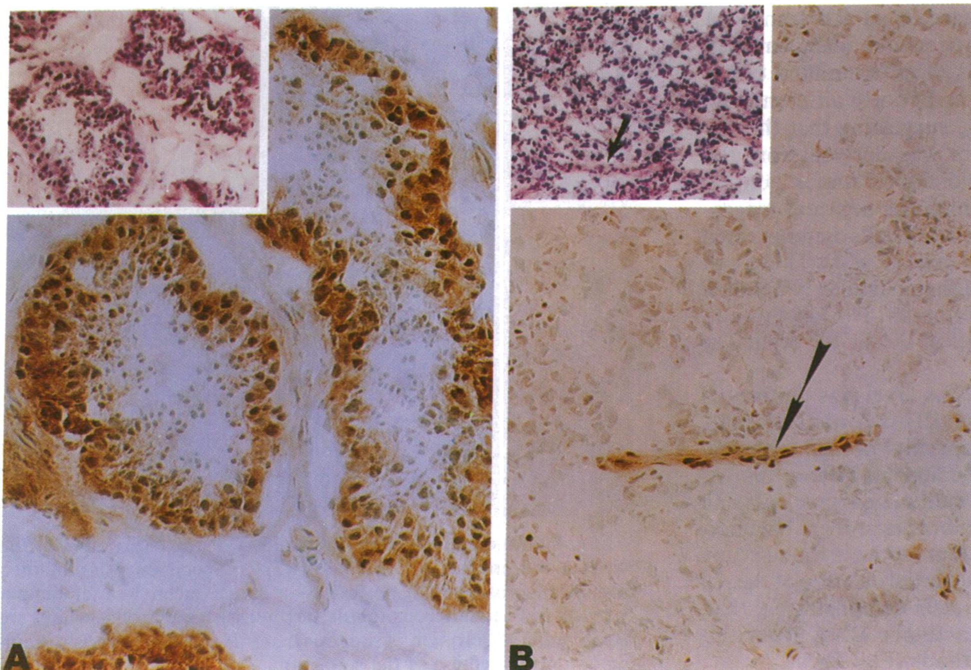

FIG. 2. Immunohistochemical analysis of the expression of the *RB*-encoded nuclear protein. (*A*) Normal testicular tissue with preserved seminiferous tubules. (×160.) RB staining in nuclei of spermatogonia, spermatocytes, and Sertoli cells and a heterogeneous RB staining pattern in the more mature spermatids and spermatozoas are shown. (*Inset*) Hematoxylin/eosin staining of a serial section of the same area. (×80.) (*B*) Seminoma GCT. (×160.) There is no nuclear staining in the tumor cells, whereas strong nuclear staining occurs in endothelial cells (arrow) and lymphocytes found in the section. (*Inset*) Hematoxylin/eosin staining of a serial section of the same area. (×80.)

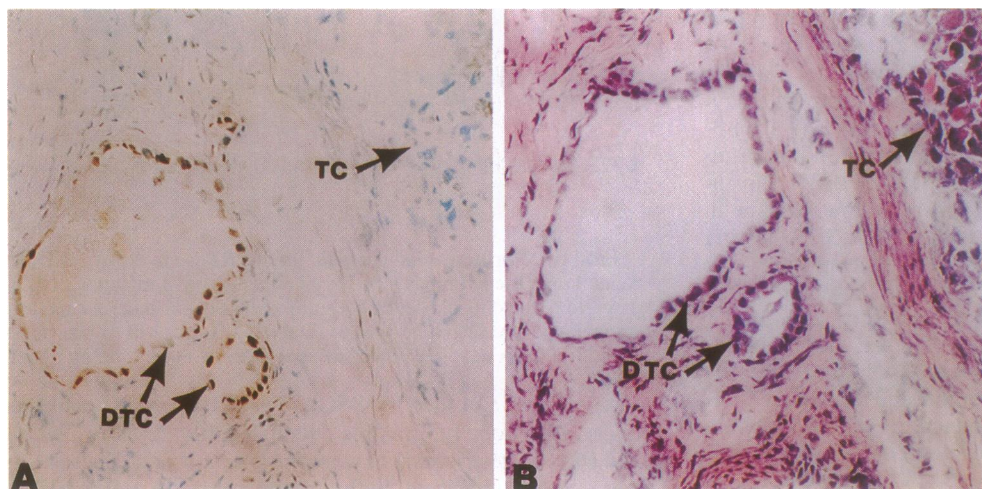

FIG. 3. Immunohistochemical analysis of the *RB*-encoded protein. (*A*) Nonseminoma GCT (teratocarcinoma subtype). (×160.) Heterogeneous protein expression was found in the tumor. Differentiated tumor cells (DTC) mimicking mature epithelium show nuclear RB staining, but the less differentiated tumor cells (TC) do not stain. (*B*) Hematoxylin/eosin staining of a serial section of the same area. (×160.)

ognizes the 105-kDa *RB* gene product in immunoprecipitation, on Western blots, and in immunohistochemical assays (28, 36, 37). Consistent with the transcript data, there was no detectable nuclear staining in any of the malignant cells of the 27 seminomas, 12 embryonal carcinomas, or two choriocarcinomas, indicating absence of the 105-kDa nuclear phosphoprotein encoded by *RB* (Fig. 2). Conversely, almost all germ cells from normal testes and normal testicular tissue adjacent to the tumors were found to have strong nuclear immunoreactivity (Fig. 2). The smaller more differentiated germ cells located in the lumen of the seminiferous tubules (spermatids and spermatozoa) showed a heterogeneous staining pattern. Normal cells within the tumor specimens such as infiltrating lymphocytes, endothelial cells, and nonmalignant stromal cells of the interstitial tissues also had positive nuclear staining (Fig. 2). Of note, in 14/15 teratocarcinomas, the malignant cells representing the more differentiated cell type (i.e., teratoma cells) stained positive for the *RB*-encoded protein, whereas undifferentiated cells found in the same tumor specimen (i.e., embryonal carcinoma cells) did not stain (Fig. 3). Moreover, differentiated teratomatous structures found in 5/11 tumors with mixed histologies expressed the protein. Intense nuclear staining was detected in the cells of mature teratocarcinoma elements differentiating into cartilage, muscle, or glandular epithelium (Fig. 3). Two of the three GCTs with 1.5- to 2-fold decrease of RB transcript levels were shown to have a heterogeneous malignant cell population consisting of large areas of differentiated tumor cells with positive nuclear staining and poorly differentiated cells that lacked immunoreactivity, suggesting that the RB transcript detected in these tumors was derived from the more mature cells of teratocarcinomatous tumor components. Sections of specimens in which the adjacent macroscopically "normal" tissue contained CIS cells demonstrated no detectable RB protein in the CIS cells (Fig. 4). There was clear nuclear staining in the cells of the testicular lymphoma and in the cells of the Leydig-cell tumors. No RB protein, however, was detected in the nuclei of the SCLC testicular metastasis (data not shown).

Analysis of the *RB* gene at the DNA level was performed by Southern blot analysis after *Hin*dIII digestion of DNA from 51 GCTs and 28 macroscopically normal adjacent testicular tissues from the same specimens. Fifteen tumor/normal pairs underwent additional multiple enzyme cuts and analysis with chromosome 13 polymorphic probes. No alterations of *RB* gene structure or copy number were found in any of the GCTs. There was, however, a partial deletion of the *RB* gene in the SCLC testicular metastasis demonstrating that, when present, gross alterations in tumor DNA from this cohort are detectable by these methods (Fig. 5).

DISCUSSION

The data from this study suggest that decreased expression of the *RB* gene is associated with the majority if not all human testicular GCTs. This decrease may occur at the transcriptional level or alternatively as a result of decreased transcript half-life leading to a commensurate decrease in RB protein expression. No alterations were found at the DNA level even when multiple enzyme cuts and hybridization with chromo-

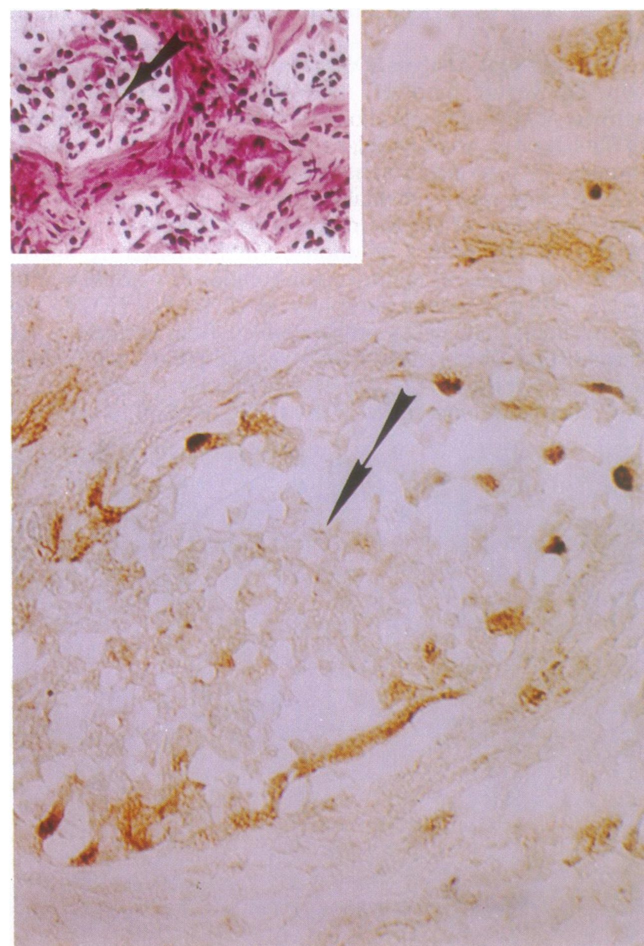

FIG. 4. Immunohistochemical analysis of the expression of the *RB*-encoded protein in CIS cells. (×320.) Specimen was obtained from the macroscopically normal tissue area adjacent to a seminoma. A preserved seminiferous tubule (arrow) with CIS cells, which are negative for RB protein, is shown. (*Inset*) Hematoxylin/eosin staining of a serial section of the same area. (×80.)

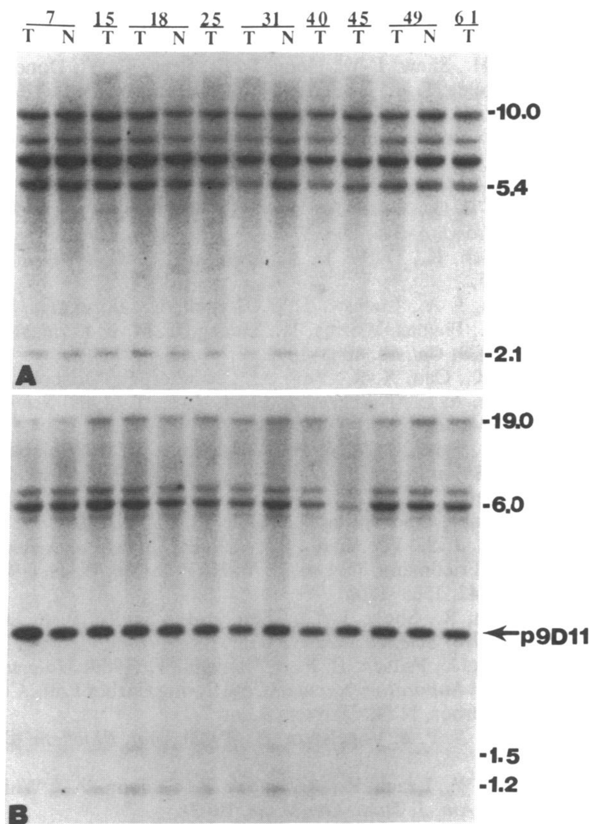

FIG. 5. Southern blot analysis of the *RB* gene in testicular GCTs. Hybridization with the 3′ fragment (*A*) and the 5′ fragment (*B*) of the RB probe. No quantitative or qualitative alterations of the gene were detected in primary testicular tumors. However, a partial deletion was found in the testicular metastasis of a SCLC (lane 45 T). Numbers above lanes are specimen identification numbers. Lanes T and N are as in Fig. 1. Positions of molecular markers (kb) are shown to the right.

some 13 polymorphic probes were performed. Structural alterations of the gene at the DNA level, however, cannot be completely excluded. Southern blot analysis is an insensitive tool for detecting subtle genetic alterations such as point mutations or small deletions. Evidence that these types of mutations occur in the *RB* gene of some human tumors has been reported. Gross structural alterations of the *RB* gene detected by Southern blot analyses were found in only a small subset of tumors (24–27, 32–34), whereas more sensitive techniques (e.g., use of PCR or ribonuclease protection assays), identified microdeletions and point mutations in a number of tumors that had normal *RB* banding patterns on Southern blot analysis (35, 50). Mutations of the *RB* gene in the human GCTs of our cohort may also be below the limits of detection by Southern blot analysis. A strong argument against this, however, is the heterogeneous expression of the protein in cells of teratocarcinomas. In the majority of these tumors the RB protein is absent in the less differentiated (embryonal) tumor cells but present in the mature (teratoma) cells of the same specimen. One explanation for this observation may be that teratocarcinomas are polyclonal in origin and that some cells have lost their ability to express the *RB* gene whereas others have not. Arguing against this, however, is evidence suggesting that all nonseminomatous GCT cells develop from a single pluripotent embryonal carcinoma cell. This evidence is derived from *in vitro* and *in vivo* models and from immunohistochemical studies of actual human tumor tissues (51, 52). It is currently thought that the pluripotent cell originates from a common precursor of both seminomas and nonseminomas, the so-called CIS cell (3–5, 51, 52).

This model of clonal origin would preclude the possibility that structural alterations in the *RB* gene account for absent transcript and/or protein in undifferentiated cells since reappearance of the protein in the more differentiated teratoma cells of the same tumors could not be explained if the gene were structurally damaged. Instead our data suggest that regulation of the *RB* gene is responsible for the changes in expression found in human testicular GCTs. To our knowledge, the *RB* gene has not been reported to be regulated at the transcript level. Earlier studies have shown that the RB protein is expressed throughout the cell cycle in primary human umbilical vein endothelial cells, HeLa, HL-60, and breast and bladder carcinoma cells and that proliferating cells do not decrease their RB protein content after growth stimulation. These and other studies (38–43) have suggested that the activity of *RB* is regulated through phosphorylation of the protein. Recent data on the structure of the *RB* promoter region, however, indicate that it has similarities to promoter regions of transcriptionally regulated genes such as the epidermal growth factor receptor (53). Moreover, studies of *RB* gene expression in lymphocytes of patients with a germline deletion of one allele suggest the possibility of transcriptional regulation (54).

Although GCTs are clinically distinct tumors, it is of interest to note that decreased RB transcript and protein levels are consistently found in poorly differentiated cells of all testicular germ-cell malignancies regardless of the histologic subtype. This finding coupled with the same observation in testicular CIS cells suggest some common mechanism(s) in the biology of these tumors. It is unlikely, however, that decreased *RB* expression would be the only event responsible for the development of testicular tumors since GCTs are rare secondary malignancies in patients with previous heritable retinoblastoma (55, 56). The precise contribution of changes in *RB* gene expression to the pathogenesis of human GCTs remains to be determined.

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