Lack of RBM Expression as a Marker for Carcinoma In Situ of Prepubertal Dysgenetic Testis

LETIZIA SCHREIBER,* BEATRIZ LIFSCHITZ-MERCER,* GEDALIA PAZ,† HAIM YAVETZ,† ZVI ZADIK,‡ KRZYSZTOF KULA,§ JOLANTA SLOWIKOWSKA-HILCZER,§ RODOLFO REY,¶ AND BATIA BAR-SHIRA MAYMON²

From the *Institute for the Study of Pathology and the †Institute of the Study of Fertility, Lis Maternity Hospital, Tel-Aviv Sourasky Medical Center, Sackler Faculty of Medicine, Tel-Aviv University, Tel-Aviv, Israel; ‡Pediatric Endocrine Unit, Kaplan Medical Center, Rehovot, Israel; §Department of Andrology and Reproductive Endocrinology, Institute of Endocrinology, Medical University of Lodz, Lodz, Poland; ¶Endocrine Research Center, R. Gutierrez Children’s Hospital, Buenos Aires, Argentina; and ¶¶Institute for Human Genetics, The International Center for Life, University of Newcastle upon Tyne, Newcastle upon Tyne, United Kingdom.

ABSTRACT: Individuals with various intersex states who carry Y-chromosome material bear a high risk of developing testicular neoplasia. In order to gain more insight into the pathogenesis of this neoplasia, the current study evaluates the differentiation of the seminiferous epithelium in 46,XY dysgenetic male pseudohermaphroditism. Immunohistochemical evaluation was performed using the germ cell-specific RNA-binding motif (RBM) protein (encoded by the Y-chromosome) to identify normal germ cells, whereas placental alkaline phosphatase (PLAP) was used to detect neoplastic germ cells. Differentiation of somatic Sertoli cells was assessed using cytokeratin-18 (CK-18) and anti-Müllerian hormone (AMH) as markers for immature Sertoli cells. Specimens were taken from surgically removed dysgenetic gonads of five children (46XY karyotype). Intra- tubular germ cell neoplasia (carcinoma in situ [CIS]) of the testis was detected in all of them. Normal germ cells revealed immunoreactivity for RBM, whereas the PLAP-positive neoplastic germ cells were negative for RBM expression. Sertoli cells revealed an immature phenotype indicated by AMH expression in their cytoplasm. The design of the current study is unique in its assessment of the state of germ cell differentiation in dysgenetic gonads by the use of the RBM protein, which was expressed only in normal germ cells but not in malignant testicular germ cell tumors nor in the preneoplastic lesion, intra- tubular germ cell neoplasia (carcinoma in situ [CIS]) of the testis. This points toward the germinal component of CIS as the precursor for the promotion of testis cancer.

Key words: male pseudohermaphroditism, testicular carcinoma in situ, germ cell differentiation, Sertoli cell differentiation, immunohistochemistry.

J Androl 2003;24:78–84

Patients with gonadal dysgenesis and various intersex states who carry Y-chromosome material are at greater risk for developing germ cell tumors. These disorders of sexual differentiation include 45, X/46, XY mosaicism, androgen insensitivity syndrome, and 46, XY/iso(p) Y mosaicism (Rosai, 1996; Ulbright, 1997).

The immunohistochemical evaluation described herein characterized the differentiation of the seminiferous epithelium in the pathological condition of 46,XY dysgenetic male pseudohermaphroditism. The seminiferous epithelium contains both somatic Sertoli cells and cells of the germ line (Russell, 1993; de Kretser et al, 1998). The germinal component can be identified by expression of the RNA-binding motif (RBM) protein, which is encoded by one or more genes located in the azoospermia factor-b (AZF-b) region on the human Y chromosome long arm (Elliott et al, 1997). The RBM protein is expressed in male germ cell nuclei of fetal (ie, from the second trimester of gestation), prepubertal, and adult testes (Elliott et al, 1997). In addition, this marker can assess the state of differentiation of male germ cells because RBM is consistently expressed in normal differentiated germ cells (Elliott et al, 1997; Bar-Shira Maymon et al, 2001), but not in malignant testicular germ cell tumors nor in the preneoplastic lesion, intra- tubular germ cell neoplasia carcinoma in situ (CIS) of the testis (Lifschez-Mercer et al, 2000, 2001).

Undifferentiated germ cells can be detected by their positive immunoreactivity to placental alkaline phosphatase (PLAP). This marker is normally expressed in primordial germ cells during embryogenesis and in gonocytes, ie, the fetal germ cell (Pedersen, 1988; Hofmann and Millan 1993; Jørgensen et al, 1995). PLAP is known to be expressed in testicular tissue only in germ cell tumors or in CIS cells in children and adults (Giwercman et al, 1991). The postnatal presence of PLAP-immuno-
positive germ cells that are similar in appearance to gonocytes is considered pathologic (Jørgensen et al, 1993, 1995).

The mode of differentiation of the somatic component, the Sertoli cell, can be assessed by expression of the markers anti-Müllerian hormone (AMH) and cytokeratin-18 (CK-18). AMH is secreted by Sertoli cells in various mammalian species, including humans (Tran et al, 1987). During embryogenesis, the production of AMH is responsible for the regression of components of the Müllerian ducts, which assures normal phenotypic development of genitalia in males (Donahoe et al, 1983; Josso et al, 1993). CK-18 is a subtype of the cytokeratin family and represents an excellent marker for epithelial differentiation (Moll et al, 1982; Miettinen et al, 1985).

AMH and CK-18 are typically coexpressed in Sertoli cell cytoplasm in the male fetus but not in adulthood (Stege et al, 1996, 1999). Expression of CK-18 is lost during prepubertal maturation (Stosiek et al, 1990; Amüller et al, 1992; Bergmann and Kliesch, 1994), whereas AMH is persistently secreted by premeiotic seminiferous tubules up to puberty (Rey et al, 1996; Steger et al, 1996, 1999).

The predominant intermediate filament expressed consistently in Sertoli cells is of the vimentin type, indicating the mesenchymal origin of these cells (Franke et al, 1979). Expression of AMH, CK-18, or both in the seminiferous epithelium is regarded as a sign of an immature Sertoli cell state, demonstrating a prepubertal stage of development (Miettinen et al, 1985; Stosiek et al, 1990; Auümüller et al, 1992; Bergmann and Kliesch, 1994; Steger et al, 1996, 1999; Bar-Shira Maymon et al, 2000, 2002).

The current study applies immunohistochemical methodology to investigate the differentiation of both components of seminiferous epithelium—germ cells and Sertoli cells—in 46,XY testicular dysgenesis. Evaluation of germ cell differentiation in dysgenetic gonads using the RBM marker has not been previously reported.

Materials and Methods

Patients

Five children aged 2–5 years from Poland were included in the study. Criteria for study inclusion were the 46,XY karyotype, ambiguous genitalia, and nonpalpable gonads. The clinical evaluation of these 46,XY dysgenetic male pseudohermaphrodite patients was previously described in detail by Słowikowska-Hilczer et al (2001); patients 2–6 of their study were evaluated in the current study. Specimens were taken from the dysgenetic gonads that had been surgically removed. CIS lesions were identified in this child, and the specimen was used to assess marker expression in a gonad that contains only normal germ cells.

Histological Evaluation

The specimens were fixed in Bouins solution and embedded in Paraplast. Histomorphological analysis was performed on hematoxylin and eosin–stained thin sections. The morphological criteria for recognizing CIS cells included a nucleus that was irregular in shape, irregular coarse clumps of chromatin, and light abundant cytoplasm (Holstein et al, 1987; Müller, 1987). The scoring methodology as well as the quantitative description of the specimens carrying CIS was previously described for patients 2-6 by Słowikowska-Hilczer et al (2001).

Immunohistochemistry

Immunohistochemistry was performed using monoclonal antibodies against CK-18 (clone DC10, Zymed, San Francisco, Calif), vimentin (clone V9, DAKO, Copenhagen, Denmark), RBM, and AMH. The methodology for generating AMH and RBM antibodies is described elsewhere (Rey et al, 1996 and Elliott et al, 1997, respectively). A monoclonal antibody against PLAP (clone 8B6, DAKO, Glostrup, Denmark) was also used. Briefly, thin sections were processed by the labeled-(Strept) avidin-biotin (LAB-SA) method using a Histostain plus kit (Zymed). Heat-induced antigen retrieval was performed by controlled microwave treatment using an H2800 model processor (Energy Beam Sciences Inc, Agawan, Mass) in 10 mM citrate buffer pH 6.0 for 10 minutes at 97°C. The sections were treated with 3% H2O2 for 5 minutes, followed by a 10-minute incubation with a universal blocker, CAS block (Zymed), for reducing nonspecific immunolabeling. Consecutive sections were incubated for 1 hour with ready-for-use CK-18 antibody, a 1:500 dilution of antivimentin antibody, a 1:500 dilution of anti-RBM antibody, a 1:1000 diluted AMH antibody, and a 1:50 dilution of anti-PLAP antibody.

A biotinylated secondary antibody was applied for 10 minutes, followed by incubation with horseradish peroxidase (HRP)-conjugated streptavidin (HRP-SA) for 10 minutes. Following each incubation, the slides were washed thoroughly with Optimax wash buffer (Biogenex, San Ramon, Calif). The immunoreaction was visualized with an HRP-based chromogen/substrate system, including diaminobenzidine (DAB; brown) chromogen (Liquid DAB substrate kit; Zymed). The sections were counterstained with Mayers hematoxylin and mounted for microscopic examination.

A gonad obtained from a 14-week-old male fetus (Maymon et al, 2000) was used as the control for antibody immunoreactivity. In this specimen, all the germ cells were immunoreactive for PLAP and lacked expression of the RBM marker. Its Sertoli cells were immunopositive for expression of CK-18, AMH, and the vimentin marker.

Negative control incubations were performed by substituting nonimmune serum for the primary antibody. All these sections were entirely immunonegative.

For double immunolabeling, the sections were first stained with the anti-RBM antibody followed by staining with the anti-PLAP antibody (according to the procedure described above)
Figure 1. (A–F) Immunohistological characterization of the normal differentiated gonad (without CIS lesions). Original magnification 400x. Arrows indicate germ cell nuclei. The arrowhead indicates Sertoli cell cytoplasm. (A) Immunohistochemical staining by the RBM antibody revealing a focus of tubules that contain germ cells. Germ cell nuclei are strongly marked. (B) Immunohistochemical staining by the PLAP antibody showing that all germ cells are entirely immunonegative. (C) Immunohistochemical staining by the AMH antibody demonstrating that Sertoli cell cytoplasm is uniformly immunoreactive. (D) Immunohistochemical staining by the CK-18 antibody demonstrating minimal staining in Sertoli cell cytoplasm.

Results

The histologic evaluation performed on hematoxylin eosin–stained sections demonstrated a high prevalence of CIS lesions in testicular dysgenesis (ie, undifferentiated germ cells were identified in all dysgenetic gonads of the five patients from Poland). All detectable germ cells were found to be normal only in the gonads of the patient from Israel with androgen insensitivity syndrome. In the latter patient with a normal differentiated gonad (without CIS lesions), the germ cells could be morphologically identified by their nuclei, which were rounder and larger than those of Sertoli cells, their pale cytoplasm, and via immunohistochemistry using RBM protein expression. The immunohistochemical evaluation of this normal differentiated gonad (Figure 1, A–D) detected the germ cells by their strong immunoreactivity for RBM in the nuclei (Figure 1A). The germ cells were detected in only a few foci located mainly in the inner part of the gonad, whereas most of the other tubules consisted of Sertoli cells only. All germ cells that were detected either by morphology or by RBM immunoreactivity revealed a differentiated phenotype and were immunonegative for PLAP expression (Figure 1B). The Sertoli cells in this normal differentiated gonad had an immature phenotype as indicated by the uniform, strong immunoreactivity for AMH in the cytoplasm of Sertoli cells (Figure 1C). CK-18 showed minimal staining in the cytoplasm of a few Sertoli cells using an AEC solution (Zymed) as a (red) chromogen to visualize the immunoreaction.
Figure 2. (A–D) Immunohistological characterization of dysgenetic gonads carrying CIS lesions. Original magnification 400×. (A) Immunohistochemical staining by the RBM antibody. Normal germ cells are identified by their positive immunoreactivity for RBM (arrow), and undifferentiated germ cells lack the markers expression (arrowheads). (B) Immunohistochemical staining by the PLAP antibody showing immunoreactivity in the cytoplasm of atypical germ cells. Note that both PLAP-positive (thick arrow) and PLAP-negative (thin arrow) atypical cells are present. (C) Immunohistochemical staining by the AMH antibody demonstrating that Sertoli cell cytoplasm is uniformly immunoreactive. Germ cells (arrow) are negative for the marker expression. (D) Immunohistochemical staining by the CK-18 antibody. The specimen is entirely negative for the marker expression.

(Figure 1D). All Sertoli cells were entirely positive for vimentin, the immunostaining confined mainly to the basal portion of the cells (data not shown).

Morphological and immunohistochemical evaluation of the five dysgenetic gonads carrying CIS lesions demonstrated a different immunohistochemical profile of germ cells. All detected undifferentiated germ cells lacked RBM protein expression (Figure 2A), whereas all the normal differentiated germ cells were RBM immunopositive (Figure 2A) as defined by morphological criteria. PLAP expression was detected in the cytoplasm of CIS cells (Figure 2B), however, not all of undifferentiated germ cells expressed the PLAP marker (Figure 2B).

The immunohistochemical profiles of Sertoli cells in the dysgenetic gonads carrying CIS lesions (Figure 2, C and D) were identical to those of Sertoli cells observed in the gonad with normal differentiated germ cells (Figure 1, C and D); that is, all detectable Sertoli cells were immunopositive for AMH (Figure 2C), CK-18 expression was barely detected (Figure 2D), and all Sertoli cells were immunoreactive for vimentin (data not shown).

Double immunolabeling demonstrated normal germ cells by immunopositive reactivity for RBM (Figure 3), whereas RBM marker expression was absent in all PLAP-positive undifferentiated germ cells (Figure 3).

Discussion

The current study is the first to assess the state of germ cell differentiation in a dysgenetic gonad using the RBM antibody. The RBM antibody is unique in its specificity to male germ cells of nonneoplastic testicular tissue (Elliott et al, 1997; Bar-Shira Maymon et al, 2001). The other markers of CIS lesions (PLAP, TRA-1-60, 43-9F, and M2A) are known to have a wide distribution in normal and neoplastic tissues (Giwercman et al, 1991, 1993). For example, anti-PLAP antiserum is raised against a placen-
tal protein, anti-TRA-1-60 antiserum is raised against embryonal carcinoma, anti-43-9F antiserum is raised against ovarian carcinoma (Giwercman et al, 1991, 1993), and anti-RBM antiserum is raised against a protein encoded by the Y chromosome, which results in restricted expression of the marker in male germ cells (Elliott et al, 1997).

RBM expression was observed at 16–18 gestational weeks and is maintained in male germ cells of differentiated testicular tissue in adults (Elliott et al, 1997). CIS cells have been occasionally detected in the testes of infants, the youngest only 2 months old, and are believed to arise prenatally from primordial germ cells and to rest in the testis until subsequent tumor development in postnatal life (Skakkebaek et al, 1984, 1987; Jorgensen et al, 1993). The CIS cells that were observed in the current study have immunohistochemical similarity to those early primordial germ cells (ie, of embryos younger than 16 weeks) in terms of the absence of RBM protein expression. This result supports the hypothesis of an early fetal origin of CIS of the testis.

CIS cells may be overlooked by routine histologic procedure because it can be difficult to identify the in situ component of germ cell neoplasia on the basis of morphology. The use of an immunohistochemical marker in addition to standard morphology can improve diagnostic sensitivity. Using the absence of RBM, which is known to be expressed exclusively and consistently only in normal differentiated testicular tissue as a marker of undifferentiated germ cells, may enhance the ability of the pathologist to identify CIS lesions (Lifschits-Mercer et al, 2000, 2001). The foci of CIS are usually in the vicinity of tubules that contain normal differentiated germ cells (immunopositive for RBM expression), and which serve as a built-in control for the reactivity and sensitivity of the RBM antibody.

The specimens of the current study were evaluated in an earlier study by Slowikowska-Hilczer et al (2001). Their immunohistochemical evaluation demonstrated that both PLAP-positive and PLAP-negative CIS cells were present in dysgenetic gonads, whereas densitometry of all these CIS cell nuclei revealed hyperploid DNA content in their nuclei (Slowikowska-Hilczer et al, 2001). Because hyperploidy is a characteristic feature of germ cell tumors (De Jong et al, 1990; Fischer et al, 1994), all CIS cells should be considered neoplastic. Importantly, expression of the RBM marker was absent in all CIS cells...
observed in the current study, even in PLAP-negative CIS cells, which might have been missed by screening with only a PLAP marker. This finding is a clear demonstration of the accuracy of the RBM marker in assessing the state of germ cell differentiation. It also indicates that the combined use of PLAP and RBM markers would enhance the diagnostic sensitivity of immunohistological evaluations in the detection of CIS lesions.

It is commonly accepted that all forms of testicular germ cell tumors (with the notable exception of spermatocytic seminoma) are derived from a common precursor, the intratubular germ cell neoplasia, or CIS, of the testis (Skakkebaek, 1972; Skakkebaek et al, 1984; Srigly et al, 1988). The results of the current study as well as the earlier findings of Slowikowska-Hilczer et al (2001) revealed a high prevalence of abnormal undifferentiated germ cells in the seminiferous epithelium of 46,XY intersex patients. This supports the hypothesis that CIS is the precursor for the development of testicular neoplasia. As an underlying etiology for the development of this neoplasia in 46,XY testicular dysgenesis, Slowikowska-Hilczer et al suggest that gonadotropins may be involved in the formation or maintenance of germ cell pathology, and as a consequence, participate in the promotion of testicular cancer (Slowikowska-Hilczer et al, 2001).

The immature phenotype of the prepubertal Sertoli cells was not affected by testicular dysgenesis or by the interrelationship with abnormal germ cells, as indicated by persistent expression of AMH in premeiotic seminiferous tubules. This phenomenon was previously demonstrated by Rey et al (1996) in patients aged 1 month to 14 years with the 46,XY karyotype who had either an- drogen insensitivity or testicular steroidogenesis disorders, persistent Müllerian duct syndrome, gonadal dysgenesis, or true hermaphroditism (Rey et al, 1996).

Sertoli cell maturation in the presence of CIS was previously studied in testicular biopsies of adult patients with testicular tumors or CIS (Kliesch et al, 1998). In their study, the tubules bearing CIS, together with normal germ cells, were negative for CK-18 expression, indicating a mature Sertoli cell phenotype (Kliesch et al, 1998). In contrast, in cases in which the tubules were entirely devoid of normal germ cells, the tubules bearing CIS showed positive CK-18 expression in Sertoli cells. This suggests that Sertoli cells in adults, which are in the vicinity of CIS cells only (not normal ones), undergo a process of dedifferentiation, as evidenced by re-expression of cytokeratin intermediate filaments (Kliesch et al, 1998).

The Y chromosome has been central to research in male infertility because several genes that might control spermatogenesis are on its long arm (Lahn and Page, 1997). The present study points to an association of the absence of expression of the Y chromosome RBM gene or genes and the presence of neoplastic germ cells. The observation that the earliest stages of germ cell neoplasia, CIS, do not express normal germ cell protein markers such as RBM, suggests that either 1) this neoplasia is derived from cells that originate early in development (before such genes are normally turned on), or 2) RBM genes have been turned off when the neoplastic cells have subsequently lost the normal germ cell differentiation pathway.

Defects in AZF genes are normally implicated as causative agents in male infertility. However, given their presumptive importance in normal germ cell development and that in many cases cancer can be considered to be development “gone wrong” (Hastie, 1994), taken together with the observation that common etiologic factors may exist for testicular cancer and male subfertility, it is possible that misexpression or nonexpression of the genes encoded by the Y chromosome AZF region might participate in the mechanisms responsible for neoplastic transformation of germ cells. Further studies are needed in order to elucidate whether RBM gene expression is associated solely with fertility functions in the adult, or whether it is an active player in the developmental control mechanisms responsible for normal differentiation of testicular tissue.

Acknowledgment

We thank H. Cooke for providing the RBM antibody.

References


