Aurora B expression in normal testis and seminomas

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Abstract

Aurora/Ipl1-related kinases are a conserved family of proteins that have multiple functions during mitotic progression. High levels of Aurora kinases are characteristic of rapidly dividing cells and tumours. Aurora B encodes a protein that associates with condensing chromatin, concentrates at centromeres, and then relocates onto the central spindle at anaphase. In this study the expression and the localisation of Aurora B throughout germinal epithelial progression in normal testis and its neoplastic counterpart were analysed.

Immunocytochemistry and RT-PCR analysis of mouse germinal epithelium cells showed the presence of Aurora B in spermatogonia and occasionally in spermatocytes. Western blot analysis revealed the typical Aurora B isoform (~41 kDa) in the same cellular types. A similar distribution was observed in human testis by immunohistochemistry. Moreover, the distribution and the expression of Aurora B were investigated in neoplasms derived from germ cells. Surgical samples of seminomas were analysed, and a high percentage of Aurora B positive cells (51%) was detected; the expression of Aurora B was significantly related to the MIB-1 proliferation marker ($R = 0{\cdot}816$).

The data presented here demonstrate that Aurora B expression occurs in spermatogonial division. Furthermore, our results indicate that the expression of Aurora B is a consistent feature of human seminomas.

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Introduction

Mitosis is a highly coordinated process that ensures the fidelity of chromosome segregation and is characterised by dramatic morphological changes which occur in a strictly sequential order (Cao & Wang 1990). The mechanisms that coordinate the cycle of chromosome condensation and decondensation with the assembly, function, and subsequent disassembly of the mitotic spindle are poorly understood. Highly conserved genes essential for chromosome condensation have been found through genetic screens in yeasts and *Drosophila* (Bhat *et al.* 1996, Sutani *et al.* 1999). Protein phosphorylation has been suggested as an important regulatory mechanism for cytokinesis. Among the protein kinases, serine/threonine protein kinase of the Aurora family (Aurora A/STK-15, Aurora B/AIM-1, Aurora C/AIK-3) are known to be required for the progression through the M phase. It has been shown that the products of the Aurora family genes are expressed in proliferating cells and are overexpressed in neoplastic cells (Tanaka *et al.* 1999). In particular, Aurora B has been found overexpressed in human cancer cells of different origin such as HeLa, Lovo, HT29, and in cell lines derived from colorectal tumours (Tatsuka *et al.* 1998). Moreover, high expression levels of Aurora B were detected in primary human colorectal cancers at various pathologic stages with a tendency to group in higher grades of malignancy defined by pathological observation (Katayama *et al.* 1999). Finally, overexpression of Aurora B in diploid human cells NHDF induced multinuclearity, which is one of the most common features of tumour cells (Tatsuka *et al.* 1998, Terada *et al.* 1998).

During the cell cycle Aurora B (AIM-1) peaks after Aurora A and is prominent at the midzone during anaphase and in postmitotic bridges during telophase. The two kinases display distinct subcellular localisations. Aurora B and its corresponding proteins, *Caenorhabditis elegans* AIR-2, *Xenopus laevis* AIRK2, and *Drosophila* IAL, have roles in cytokinesis (Terada *et al.* 1998). Moreover, double-stranded RNA interference analysis has revealed that Aurora B plays a critical role in chromosome segregation as well as in cytokinesis (Adams *et al.* 2001).

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Spermatogenesis is a hormonally regulated and unique developmental process whereby diploid stem cells differentiate through an ordered sequence of steps into haploid spermatozoa, highly specialised in structure and function. This process can be divided into mitotic, meiotic, and postmeiotic phases that are synchronised and are an ideal model for studying the control of cellular growth and differentiation. The molecular mechanisms that regulate and coordinate the expression of genes throughout spermatogenesis in vertebrates are not completely understood (Sharpe 1994, Sassone-Corsi 1997).

The aim of this study is to clarify the spatio-temporal localisation of Aurora B kinase in the mouse germinal epithelium progression, and its expression in normal human testis and in testicular neoplastic lesions. Our results document that Aurora B is detected in the germinal epithelium in the spermatogonia and occasionally in spermatocytes. In addition, these data support the notion that Aurora B is involved in germ cell proliferation of normal and neoplastic testis. A high percentage of Aurora B-positive cells in seminomas was detected and the expression of Aurora B was significantly related to the MIB-1 proliferation marker.

Materials and Methods

Preparation of testicular cells

Germ cells were prepared from testes of adult CD1 mice (Charles River Italia, Calco, Italy). Testes were removed from the albuginea membrane and digested for 15 min in 0·25% (w/v) collagenase (type IX, Sigma) at room temperature under constant shaking. They were then washed twice in Minimum Essential Medium (Life Technologies, Inc.), seminiferous tubules were cut into pieces with a sterile blade and further digested in Minimum Essential Medium containing 1 mg/ml trypsin for 30 min at 30 °C. Digestion was stopped by adding 10% fetal calf serum and the germ cells released were collected after sedimentation (10 min at room temperature) of tissue debris. Germ cells were centrifuged for 13 min at 1500 r.p.m. at 4 °C and the pellet resuspended in 20 ml elutriation medium (120·1 mM NaCl, 4·8 mM KCl, 25·2 mM $NaHCO_3$, 1·2 mM $MgSO_4$ ($7H_2O$), 1·3 mM $CaCl_2$, 11 mM glucose, 1 × essential amino acid (Life Technologies, Inc.), penicillin, streptomycin, 0·5% bovine serum albumin). Pachytene spermatocyte and spermatid germ cells were obtained by elutriation of the unfractionated single cell suspension as described elsewhere (Meistrich 1977). Homogeneity of cell populations ranged between 80 and 85% for pachytene spermatocytes, 95% for spermatids, and was routinely monitored morphologically. Mature spermatozoa were obtained from the cauda of the epididymus of mature mice as described previously (Sette *et al.* 1997). Spermatogonia were obtained from prepubertal mice as previously described (Grimaldi *et al.* 1993, Rossi *et al.* 1993).

The NIH-3T3 mouse fibroblasts used in these studies were cultured as recommended by the American Type Culture Collection (Rockville, MD, USA).

Tissue samples

As a source of normal tissue, ten CD1 adult mice were killed and the testes were removed and fixed in Bouin's fluid. Samples were serially dehydrated in ethanol and cleared in xylene before processing on a commercial automated tissue processor. The animals used in the present study were maintained at the Department of Biology and Pathology Animal Facility. The animal experimentations described herein were conducted in accordance with accepted standards of animal care and in accordance with the Italian regulations for the welfare of animals used in experimental studies. The study was approved by our institutional committee on animal care. As a source of neoplastic tissues, paraffin-embedded blocks from ten cases of classic seminoma were retrieved from the files of the Department of Bio-Morphological Sciences at the University 'Federico II' of Naples on the basis of the Faculty of Medicine and Surgery Ethical Committee approval; in all instances, the diagnosis was confirmed on review.

Immunohistochemistry and quantitative analysis

In normal and neoplastic testis the cellular distribution of Aurora B protein was assessed by the polyclonal antibody raised in rabbits (no. 611082; BD Transduction Laboratories, San Diego, CA, USA). The conditions of pretreatment of the histological samples most suitable for Aurora B staining have been determined by preliminary experiments on colon cancer paraffin-embedded sections that express high levels of this protein (Bischoff *et al.* 1998). We observed that regardless of the length of fixation, a proceeding step of the heat-induced antigen retrieval technique yielded a larger number of cells with specific Aurora B nuclear staining (data not shown). Based on these observations, xylene-dewaxed and alcohol-rehydrated paraffin sections were placed in Coplin jars filled with a 0·01 M tri-sodium citrate solution, and heated for 3 min in a conventional pressure cooker. After heating, slides were thoroughly rinsed in cool running water for 5 min. They were then washed in Tris–buffered saline (TBS) pH 7·4 before incubating overnight with the Aurora B (1:1000) antibody. The incubation with the primary antibody was followed by incubation with biotinylated anti-mouse immunoglobulins and by peroxidase-labelled streptavidine (LSAB-DAKO, Copenhagen, Denmark); the signal was developed by using diaminobencidine chromogen as substrate. Negative controls were run

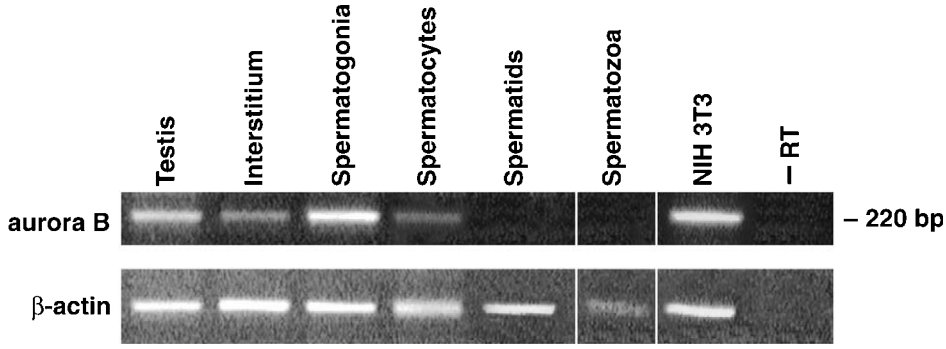

Figure 1 Expression of Aurora B mRNA in mouse testis. RT-PCR analysis of aurora B mRNA in adult mouse testis (lane 1), interstitium (lane 2), normal mouse testis germ cells (lane 3–6), NIH-3T3 cells as a positive control (lane 7), and without reverse transcriptase (− RT, lane 8). Total mRNA was then extracted, reversed transcribed and subjected to PCR analysis using primers (see Materials and Methods). The integrity of mRNA samples was determined by using β-actin as control (lower frame). The RT-PCR analysis is representative of three separate experiments.

with normal mouse serum instead of the primary antibody or the antibody was preabsorbed with the cognate peptide (10^{-6} M).

In order to assess the relationship between Aurora B expression and cell proliferation, seminomas were also stained with the MIB-1 antibody. The antibody (Immunotech, Marseille, France; diluted 1:50) recognised Ki-67, a nuclear protein expressed in the G1, S and G2/M phases; this antibody, known to stain spermatogonia, was also used as a control of antigenic preservation and of successful antigenic retrieval. Labelling indices for Aurora-B and MIB-1 antibodies were determined in the same manner. Adjacent sections were used and counting was performed in similar areas. Quantitative analysis performed with a computerised analyser system (CAS 200, Becton Dickinson, Chicago, USA) was used to score the nuclei of individual cells for expression of Aurora B and MIB-1 proteins. As already described, nuclear boundary optical density and antibody threshold were adjusted for each case examined (Bischoff *et al.* 1998). A minimal threshold was established by counting at least 1000 neoplastic cells per seminoma sample. In each case the distributions of Aurora B and MIB-1 proteins were evaluated and expressed as percentages of the total cell population. Data are reported as median and ranges. Statistical analysis was performed by means of the SPSS Inc. package (SPSS, Chicago, IL, USA). The relationship between Aurora B and MIB-1 immunostaining was analysed by calculating the nonparametric Spearman R coefficient. A P value less than 0·05 was considered significant.

Protein extraction and Western blot analysis

Mouse testes were homogenised directly into lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton-X-100, 1 mM phenylmethylsulphonyl fluoride, 1 μg aprotinin, 0·5 mM sodium orthovanadate, 20 mM sodium pyrophosphate). The lysates were clarified by centrifugation at 14 000 × **g** for 10 min. Protein concentrations were estimated by a Bio-Rad assay (Bio-Rad, München, Germany), and boiled in Laemmli buffer (Tris–HCl (pH 6·8) 0·125 M, SDS 4%, glycerol 20%, 2-mercaptoethanol 10%, Bromophenol Blue 0·002%) for 5 min before electrophoresis. Proteins were subjected to SDS-PAGE (10% polyacrylamide) under reducing conditions. After electrophoresis, proteins were transferred to nitrocellulose membranes (Immobilon Millipore Corporation, Bedford, MA, USA); complete transfer was assessed using prestained protein standards (Bio-Rad, Hercules, CA, USA). After blocking with TBS-BSA (25 mM Tris, pH 7·4, 200 mM NaCl, 5% bovine serum albumin), the membrane was incubated with the primary antibody against Aurora B (1:400; #611082, BD Transduction Laboratories) and α-tubulin (1:500; clone DM1A, Sigma-Aldrich, Milan, Italy) for 1 h (at room temperature). Membranes were then incubated with the horseradish peroxidase-conjugated secondary antibody (1:10 000) for 45 min (at room temperature) and the reaction was detected with an ECL system (Amersham Life Science, UK).

RNA extraction and RT-PCR

Total RNA was extracted from cells and tissue using the RNAzol kit (Tel-Test, Inc., Friendswood, TX, USA) according to standard procedures. Total RNA (1 mg) was extracted from specimens (Tel-Test, Inc.) and reverse transcribed (Life Technologies Italia srl). cDNA was amplified using Aurora B specific primers. The primers used were as follows: Aurora B forward: 5′-TTG ACA ACT TTG AGA TTG GG-3′; Aurora B reverse: 5′-GCT GGT CGT AGA AGT AGT TGT-3′. Expression of the

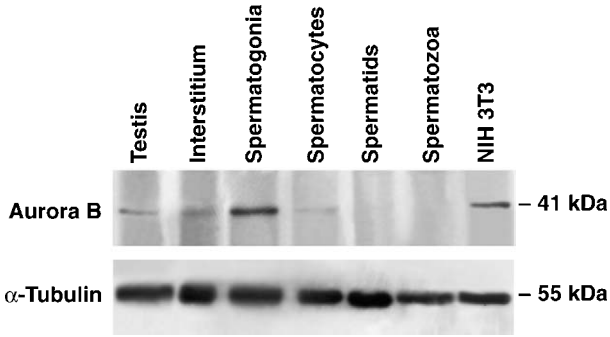

Figure 2 Distribution of Aurora B protein in mouse testis. Western blot analysis of aurora B protein in mouse adult testis (lane 1), interstitium (lane 2), normal mouse testis germ cells (lane 3–6), and NIH-3T3 cells as a positive control (lane 7) (40 µg/lane). Whole lysates were detected by Western blotting with anti-Aurora B polyclonal serum. The specific band of about 41 kDa was identified by comparison with comigrating size markers (Bio-Rad, Melville, NY, USA). α-Tubulin (Sigma) was used to assess the equal amounts of protein. The blots are representative of three separate experiments.

β-actin gene was utilised as an internal control for the amount of cDNA in the PCRs by using the following primers: β-actin forward: 5′-GTC AGG CAG CTC ATA GCT CT-3′; β-actin reverse: 5′-TCG TGCGTG ACATTA AAG AG-3′. PCR amplification was performed at 94 °C (1 min), 52 °C (1 min), and 72 °C (1 min) for 30 cycles. Reaction products were subjected to agarose gel electrophoresis. Samples amplified without previous reverse transcription were used to confirm that the signal was not due to amplification of contaminating DNA (data not shown).

Results

Aurora B expression in the mouse testicular cells

Aurora B expression in various mouse testis cells was evaluated by the RT-PCR method. Aurora B mRNA was expressed in germ cells almost exclusively in spermatogonia (spg) with a faint band present in spermatocytes (spc) (Fig. 1); a band was also detected in the interstitial cells (Fig. 1).

Western blot analysis of whole mouse testis and cell extracts from fractionated adult testis cells showed a single product migrating as a 41 kDa protein (Fig. 2). Among germ cells, it was abundant in spg, present in a few spc, and absent in spermatids (spt) and spermatozoa (spz), in agreement with the RT-PCR results. Aurora B protein was also present in the interstitial extract cells indicating a proliferative activity of these cells (Fig. 2).

Immunohistochemical analysis of Aurora B protein in mouse testis

Immunocytochemistry was performed on serial mouse testis sections using an antibody against Aurora B protein.

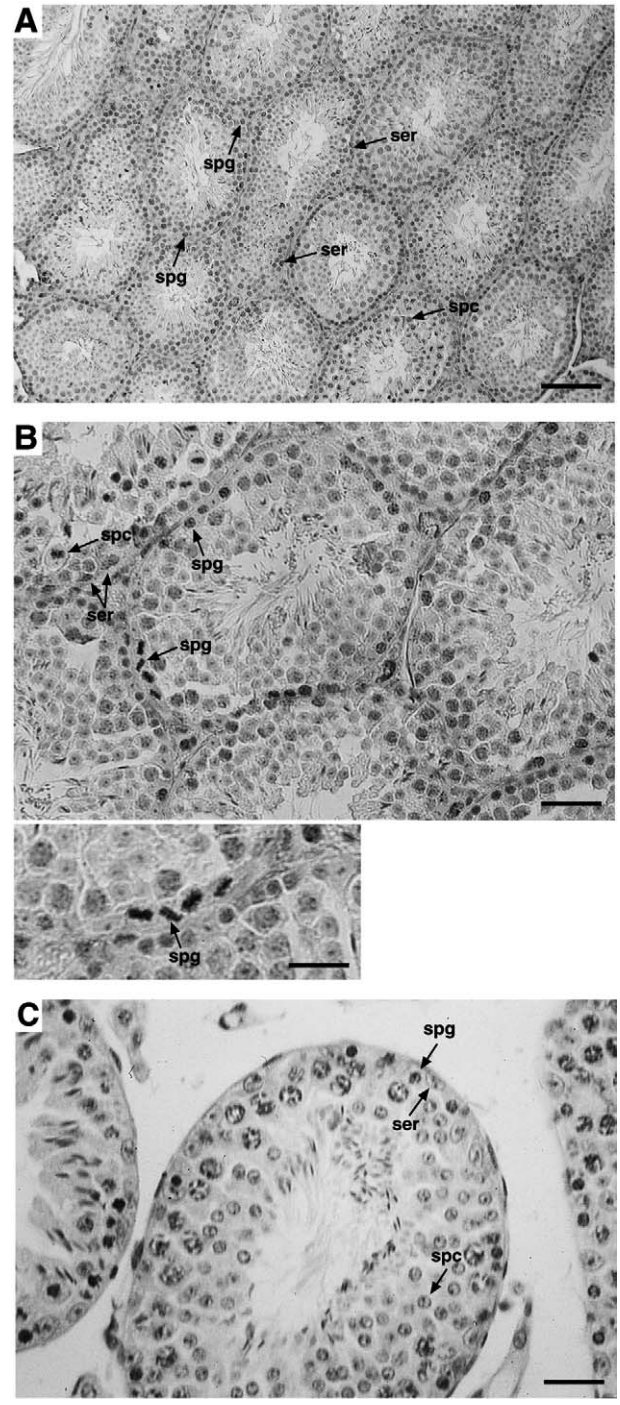

Figure 3 (A) Localisation of the Aurora B protein in sections of adult mouse testis by immunocytochemistry. Representative seminiferous tubules show staining in the spermatogonia (spg) and spermatocytes (spc). ser, Sertoli cells. Bar=100 µm. (B) Higher magnification showing specific staining for Aurora B in spg, spc and Sertoli cells (ser). Bar=50 µm. Lower panel: spg in mitotic metaphase showing Aurora B immunostaining. Bar=25 µm. (C) Control section using the antibody preabsorbed with the cognate peptide (10^{-6} M); symbols are as indicated above. Bar=50 µm.

A

	Ki-67	Aurora B
Number of cases	10	10
mean	62	46
median	66	51
range	41 - 79	29 - 55

B

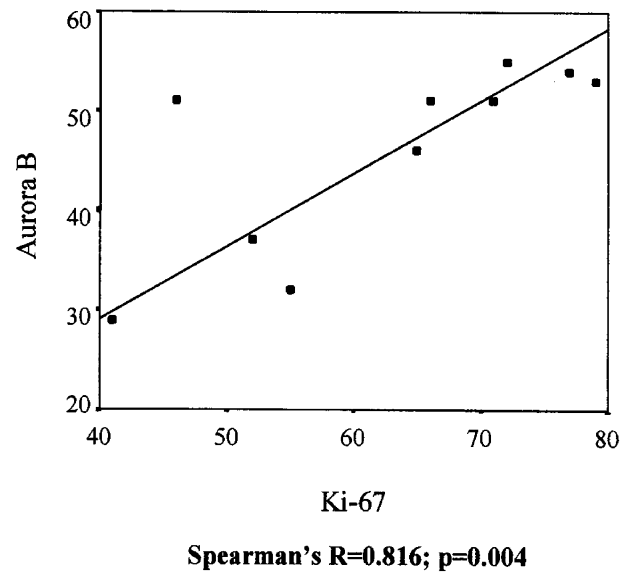

Figure 4 (A) Analysis of Ki-67 and Aurora B expression in seminomas. Values are reported as percentage of immunopositive cells. (B) Spearman analysis of the relationship between Ki-67 and Aurora B.

A topological regular expression of Aurora B was observed in seminiferous tubules with complete spermatogenesis (Fig. 3A). The protein was found in the spg and occasionally in spc (pachytene stage) and Sertoli cells (ser, Fig. 3B). In particular, immunoreactive spg showed intense metaphase mitotic figures (Fig. 3B, lower panel). On the other hand, Aurora B expression was not detectable in maturing germ cells. In fact, no labelling was seen in many primary and secondary spc, spt and spz (Fig. 3B).

The antiserum used in this study fulfils the criteria of specificity. In particular, immunoabsorption tests revealed that the labelling was quenched by preincubating antibody with 10^{-6} M of the cognate peptide (Fig. 3C).

Aurora B expression in the normal human testis and in seminomas

The vast majority of testicular cancers arise from germ cells. Premeiotic germ cells have been suggested to be the precursor for male germ cell tumours; therefore Aurora B expression was examined in a series of seminomas (Chaganti *et al.* 1994). Immunohistochemical analysis was performed in 10 cases of 'classical type' seminomas. In all cases examined a variable percentage of the neoplastic population was stained (range 29–55) as shown in Fig. 4A. The pattern of Aurora B staining in seminomas differed significantly from the pattern observed in mouse testis. Also, in the non neoplastic tissue adjacent to the neoplasia the staining pattern was limited to the basal spg and a few spc, with maturing cells being devoid of signal (Fig. 5A). As shown in Fig. 3, Aurora B-positive cells were irregularly distributed throughout the neoplastic tissue with a strong signal (Fig. 5B). Interestingly, individual dividing cells showed the most intense signal (Fig. 5B, insert).

We next compared the expression in seminomas of Aurora B to Ki-67, a well known proliferation marker, by using MIB-1 antibody. Similar to Aurora B, MIB-1 antibody was also more evident in tumours than in adjacent areas, with the percentage of Ki-67 stained cells ranging from 41–79 (Fig. 4A). Taking into account the overall data, Aurora-B expression showed a highly statistical correlation with Ki-67 ($R=0{\cdot}816$; $P=0{\cdot}004$, Fig. 4B).

It is noteworthy that the percentage of Aurora-B-positive cells was lower than the Ki-67-positive cell population, indicating that a subset of Ki-67-positive cells is also positive for Aurora B. In fact, Ki-67 was expressed by the very large majority of tumour cells (median percentage 66%), whereas the median percentage of Aurora B-positive cells was 51% of the total tumour cells (Fig. 4A).

Discussion

Spermatogenesis in mammals is characterised by a well-defined sequence of mitotic and meiotic divisions that lead to the production of mature spermatozoa. However, testicular development and normal spermatogenesis require specialised molecular mechanisms that ensure stringent stage-specific gene expression (McCarrey 1993, Chieffi *et al.* 2002).

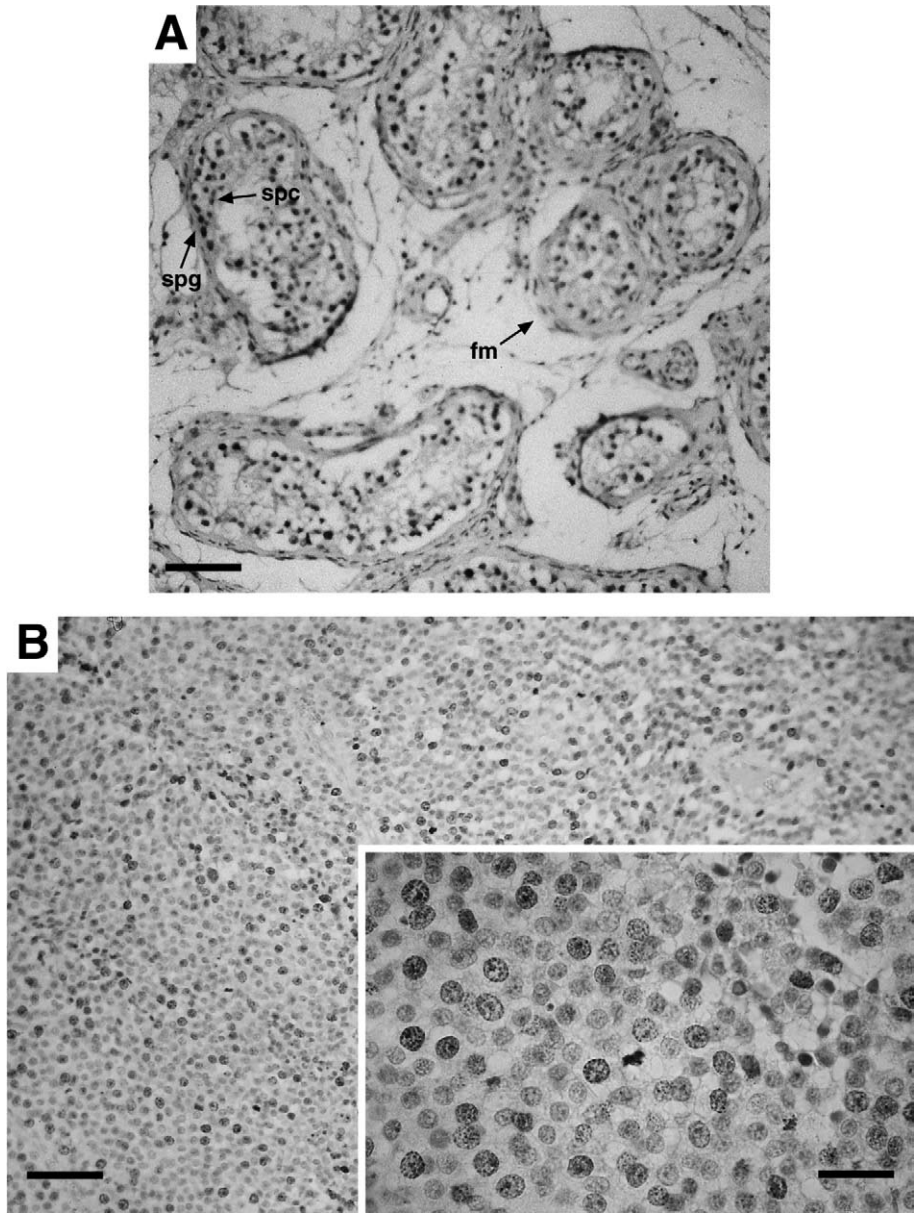

Figure 5 (A) Localisation of the Aurora B protein in sections of normal human testis by immunocytochemistry. Representative seminiferous tubules show staining in the spg and spc whereas stromal cells and fibromyocytes (fm) are devoid of signal. Bar=75 µm. (B) Paraffin-embedded section of seminoma (seminoma #6) showing extensive immunoreactivity for Aurora B protein. Bar=75 µm. Insert: at high magnification note the presence of a clear signal in neoplastic germ cells. Bar=25 µm.

In this study we have characterised the expression and the localisation of Aurora B in the testis and have demonstrated that mRNA and protein are present strongly and specifically in mitotic mouse germinal and interstitial cells, and absent in the meiotic and post-meiotic lineage cells.

Testicular tumours are rare, comprising 2% of all cancers in men; however, testicular cancer is the most common malignancy affecting males aged 20–35 years (Senturia 1987, Leendert *et al.* 1999). Because the molecular basis of these cancers needs to be elucidated, identification of cellular genes involved in testicular tumorigenesis could

increase our understanding of the development of testicular tumours, thus providing the basis for new targeted therapies.

There are at least three Aurora–related kinases in mammals, Aurora A (STK-15), Aurora B (AIM-1), and Aurora C (STK-13). All three mammalian members of this family are overexpressed in human cancer cells. Aurora A (STK-15) has been found to be overexpressed in half of all colorectal and breast cancers (mapped to 20q13) and it is highly expressed in the meiotic testicular cells. Aurora A (STK-15) is predominantly localised to centrosomes and its overexpression is supposed to be related to multiple centrosome production (Yanai *et al.* 1997, Bischoff *et al.* 1998, Giet & Prigent 1999, Zhou *et al.* 1998). Aurora C (STK-13) is also overexpressed in colorectal cancers (Takahashi *et al.* 2000).

Overexpression of Aurora B (AIM-1) has been observed in human neoplastic cell lines and in colorectal tumours (Tatsuka *et al.* 1998, Katayama *et al.* 1999). Recently, Aurora B has been shown to phosphorylate histone H3 at Ser-10 during mitosis *in vivo* (Ota *et al.* 2002). Expression of a kinase-negative form of Aurora B in Chinese hamster embryo cells (CHE) suppresses mitotic phosphorylation of histone H3 at Ser-10 (Terada *et al.* 1998). Mitotic phosphorylation of histone H3 at Ser-10 is critical for proper chromosome condensation and segregation (Terada *et al.* 1998). This has been demonstrated in *Tetrahymena* by abnormal chromosome segregation in a histone H3 mutant unable to undergo Ser-10 phosphorylation (Wei *et al.* 1999). Recent experiments performed by Sassoni-Corsi and coworkers clearly showed that Aurora B physically interacts with the H3 tail (Crosio *et al.* 2002); these data are of particular interest since the amino-terminal tail of histone H3 is able to bind its mitotic kinase from *Xenopus* egg extract (de la Barre *et al.* 2000). It has been clearly demonstrated that Aurora B overexpression induces increased mitotic H3 phosphorylation at Ser-10 (Ota *et al.* 2002), and phosphorylation imbalance induced by Aurora B overexpression might explain the errors in cytokinesis responsible for the appearance of multinuclear cells (Tatsuka *et al.* 1998). It is noteworthy that a significant overexpression of Aurora B has been observed in human cancer cell lines and in colorectal tumours in these lesions elevated expression was clearly observed in the neoplastic mitotic cells and Aurora B expression levels were increased as a function of Dukes' stage. (Tatsuka *et al.* 1998, Katayama *et al.* 1999)

In the present study, we show that Aurora B expression is a consistent feature of human seminomas, where its topological staining pattern is lost. The increase in Aurora B expression showed a positive correlation with the Ki-67 protein. It is important to note that Aurora B-positive staining in human seminomas does not completely overlap with the expression of Ki-67. The expression of Ki-67 occurs in all phases of the cell cycle excluding G0; therefore, Ki-67 immunostaining identifies all proliferating cells. Conversely, immunostaining against Aurora B may be capable of identifying, with more specificity, those cells progressing to G2/M, beyond the restriction point, indicating that Aurora B staining could provide different biological and clinical information. However, further experiments are required to assess the different expression of Aurora B and other proliferative markers.

Aurora B expression levels are regulated at both the mRNA and protein levels, with maximal mRNA and protein levels occuring during the G2/M phases (Terada *et al.* 1998). This cyclical pattern of regulation is conserved in cancer cells (Tatsuka *et al.* 1998). This indicates that the effects of Aurora B overexpression may be of critical importance during the G2/M phases of the cell cycle. The sequence of these events is essential for normal mitotic cell division in spermatogenesis and clearly overexpression of Aurora B may have implications for the abnormalities in germ cell maturation, such as those that occur in testicular cancer. Taken together, our data support a role for Aurora B in testicular mitotic germ cell maturation and, possibly, in the initiation and/or progression of testicular cancers.

In conclusion, we have shown that Aurora B expression occurs during spematogonial proliferation; in addition, expression of Aurora B is a consistent feature of human seminomas. This observation is of clinical interest since Aurora B might be a target for cancer treatment and may serve as a prognostic marker.

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