Histol Histopathol (2010) 25: 1371-1377

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Expression of mitotic kinases phospho-aurora A and aurora B correlates with clinical and pathological parameters in bladder neoplasms

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Summary. Aurora A and Aurora B are serine-threonine kinase proteins which have both been implicated in human carcinogenesis through development of aneuploidy and chromosomal instability. The aim of the study is to assess the correlation of both markers with clinical and pathological parameters in patients with bladder cancer of different grade and stage. A bladder cancer cell line was assessed for Aurora A and Aurora B expression by Western blotting. Furthermore, 85 consecutive cases of bladder neoplasms obtained by transurethral resection were quantitatively and qualitatively analysed by immunohistochemistry for Phospho-Aurora A and Aurora B expression. All cases were stratified in 4 groups according to intracellular localization (nuclear, cytoplasmic) of both markers. The association between each group and clinical and pathological parameters was assessed by statistical analysis. Higher expression of cytoplasmic Phospho-Aurora A correlated significantly with poor histological differentiation (G3 vs. G1) and advanced stage ($p<0.05$); there was also high significant correlation between nuclear Aurora B and both grading (both G3 and G2 vs. G1) and mitotic index ($p<0.05$). No statistically significant association was found between protein levels detected in tumour and sex or age ($p>0.05$). To our knowledge, the present study is the first to highlight the existence of a statistical association between such markers and traditional prognostic factors in bladder cancer. These findings indicate that Aurora A and B could be involved in the tumorigenesis of bladder cancer, thus providing a basis for a target therapy approach by using specific anti-mitotic agents.

Key words: Aurora kinases, Bladder neoplasms, Clinicopathologic parameters

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Introduction

Bladder cancer is the fourth most frequent cancer among men in Europe, with an annual incidence rate of 32/100,000 (Ferlay et al., 2001). Well-known risk factors, such as cigarette smoking and exposure to aromatic amines, taken together account for three-quarters of all cases in the Western world (Spiess and Czerniak, 2006). From a molecular point of view, several genes have been implicated in bladder carcinogenesis, mostly oncogenes (i.e. k-ras) and tumour suppressor genes (i.e. p53, pl6INK4a), whose alterations lead to genetic instability and eventually to full development of histologically detectable urothelial lesions (Spiess and Czerniak, 2006).

As a cause of genomic instability through loss of mitotic regulation and aneuploidy, Aurora A and Aurora B proteins play an important role in solid tumour development and aggressiveness (Bolanos-Garcia, 2005). They belong to the Aurora family of serine-threonine kinases, which are involved in the proper evolution of mitotic event by regulating chromosome segregation, spindle formation, centrosome separation and cytokinesis (Bolanos-Garcia, 2005). Moreover, Aurora A showed regulatory functions on the p53 pathway by affecting its DNA binding, transactivation activity and MDM2-mediated degradation, also in bladder tumour samples (Katayama et al., 2004), and

Aurora B is associated with survivin, a novel inhibitor of apoptosis and tumour-related protein, since they both belong to the chromosomal passenger group of proteins, along with INCENP and Borealin (Dasra-B), and share the same pattern of localization (Chen et al., 2003).

While Aurora A has been found to be amplified in bladder cancer, with higher levels being detected in most aggressive neoplasms showing disease progression (Compérat et al., 2007), only one paper so far has addressed Aurora B expression in urothelial neoplasms (Bruyere et al., 2008).

Therefore, the aim of the present study, which retrospectively investigates a series of consecutive cases of bladder neoplasms, is to evaluate Aurora B and the activated (phosphorylated) form of Aurora A in a series of bladder urothelial neoplasms, and to assess their association with clinical and histopathological parameters.

Materials and methods

Patients

A consecutive series of 85 bladder tumours (27 PUNLMP, 20 low grade carcinoma, pTa, 10 high grade carcinoma, pTa, 9 low grade carcinoma, pT1, 4 high grade carcinoma, pT1, 13 high grade carcinoma pT2, and 2 papillomas) obtained by transurethral resection (TUR) was analysed. All tumour diagnoses were reviewed by the same pathologists (FS, PB). Inclusion criteria were histopathologic diagnosis of urothelial tumours, availability of clinical data and of paraffin-embedded tissue specimens. Tumour stage and grade were assigned according to the TNM system (Sobin and Wittekind, 2002) and World Health Organization classifications (Sauter et al., 2004). The mitotic index was assessed in the H&E stained slides by determining the number of mitotic figures per high power field (400X). The clinicopathological characteristics of all study patients are summarized in Table 1. Muscle-invasive disease underwent cystectomy, and no stage shift or node-positive disease was noted. All pT1G3 cases underwent a subsequent bacillus Calmette-Guerin (BCG) treatment according to the European Association of Urology guidelines (Oosterlinck et al., 2006).

Table 1. Summary of clinicopathological parameters.

Age: ≤68/>68 yrs (%)	29 (34.1)/56 (65.9)
Gender: male/female (%)	76 (89.4)/9 (10.6)
Pathological T stage (%)	
non-invasive (pTa)	59 (69.4)
invasive (pT1-T2)	26 (30.6)
Pathological grade (%)	
G0	2 (2.4)
G1	27 (31.8)
G2	29 (34.1)
G3	27 (31.7)

Cell lines

The SG65 cell line was obtained from a high-grade metastatic bladder cancer; the NHEK normal human epithelial keratinocytes line was obtained from Interlab Cell Line Collection, Genova, Italy. All cell lines were cultured at 37°C in Dulbecco's Modified Eagles Medium (DMEM), supplemented with 10% foetal bovine serum (FBS), 0.075% sodium bicarbonate, 0.6 mg/ml L-glutamine. The bladder cancer cell line was kindly supplied by Prof. A. Mariggiò, University of Bari.

Western blotting

Expression levels of both Aurora B and Phospho-Aurora A proteins were determined by immunoblotting. Cells were washed twice in PBS and lysed in RIPA lysis buffer containing protease and phosphatase inhibitors. Protein quantification of the lysates was done by Bradford's method (1976). Equal amounts of protein extracts were separated on 12,5% SDS-PAGE using a Mini V-8.10 Vertical Gel Electrophoresis System (Gibco BRL, Gaithersburg, MD, USA). The Precision Plus Protein standard All Blue (Bio-Rad Laboratories, USA) was used to determine protein size. After electrophoresis, the proteins were transferred onto nitrocellulose membranes (Whatman, Schleicher & Schuell, Germany) and non-specific binding blocked by 5% w/v non-fat dry milk in TBST buffer (50 mM Tris-HCL 0.2M NaCl, pH 7.6, 0.1% Tween-20) at room temperature for 1 hour with gentle agitation, and then washed three times for 5 min each with TBST. Rabbit polyclonal anti-Aurora B kinase antibody (NB100-294, Novus Biologicals; 1:2000) and anti-Phospho-Aurora A (Thr288)/Aurora B (Thr232)/Aurora C (Thr198) (Cell Signaling; 1:1000) in TBST buffer with 5% BSA at 4°C overnight with gentle agitation were applied. After washing with TBST, the membranes were incubated with secondary antibody conjugated to peroxidase (1:10000, Pierce Biotechnology, USA) in TBST buffer with 5% BSA for 1 hour.

Western blots were developed using an ECL system (Amersham Biosciences, USA), and visualized by autoradiography. ß-actin (1:5000, Santa Cruz Biotechnology, USA) was used as a protein loading control. For protein quantification analysis, an image analyzer system was used. Negative controls without primary antibodies were performed for all samples.

Immunohistochemistry

Bladder specimens were fixed in 10% buffered formalin, then dehydrated and embedded in paraffin; 4 micron-thick sections retrieved from tissue blocks were deparaffinized in xylene and rehydrated through a graded series of ethanol solutions. Protein expression of Aurora A and Aurora B was assessed by LSAB-HRP (linked streptavidin-biotin horseradish peroxidase) immunohistochemistry procedures using primary polyclonal antibodies anti-Phospho Aurora A (T288,

Bethyl Labs., dilution 1:200) and anti-Aurora B (NB100-294, Novus Biologicals, dilution 1:3000). The number of stained cells and intensity of staining of each marker were independently scored and recorded by two observers (GP, FS), without knowledge of clinical data, to evaluate the inter-observer variation by the Cohen's K test (Software SPSS Vers 14.0). Phospho-Aurora A (P-Aurora A) and Aurora B expression were scored as percentage of stained cells (counted in at least 10 HPF), and cytoplasmic and nuclear localization were recorded separately (P-Aurora A/cyt, P-Aurora A/nucl, Aurora B/cyt, Aurora B/nucl) for subsequent statistical analyses.

Statistical analysis

The data were analyzed by the Stanton Glantz statistical software 3 (MS-DOS) and Graph-Pad Prism software version 4.00 for Windows (Graph Pad software San Diego California, USA; www.graphpad.com). Differences between groups were determined using the one-way analysis of variance (ANOVA) and the Student-Newman-Keuls test to evaluate the correlation between nuclear and cytoplasmic expression of P-Aurora A and Aurora B and clinicopathological parameters. P values <0.05 were regarded as significant.

Results

Western blot analysis

Western blot analysis for P-Aurora A and Aurora B displayed clear bands without background, of the correct molecular weight (P-Aurora A: 48 kDa, Aurora B: 39 kDa) in the bladder cancer cell line; these findings were confirmed by positive and negative controls. Activated Phospho-Aurora kinases A, B, and C showed up-regulation in SG65 cell line derived from aggressive bladder cancer compared to normal epithelial cell line, normalised for ß-actin quantity for each lane (Fig. 1). In particular, there was a 65-fold increase in SG65 cells for P-Aurora A, an 85-fold increase for P-Aurora B and a 75-fold increase for P-Aurora C (statistically significant comparison, $p<0.05$).

Immunohistochemistry

Examination of the specimens revealed focal normal urothelial cells displaying no or weak cytoplasmic positivity, as well as in the two cases of urothelial papilloma (G0). Both proteins examined showed distinct immunoreactivities, with moderate to strong cytoplasmic and/or nuclear localization (Figs. 2,3) in urothelial tumours of different grade, stage and mitotic index (Figs. 4-6). Stromal tissue remained unstained in all cases.

The immunoreactivity of both proteins was greater in cytoplasms, with no distinct pattern of localization within the urothelium (i.e. basal cells vs. superficial cells) nor within the cytoplasm (i.e. perinuclear or perimembraneous) (Table 2). An increasing trend of P-

Table 2. Summary of mean ± SE nuclear and cytoplasmic p-Aurora A and Aurora B immunoreactivity values.

	P-Aurora A/cyt	P-Aurora A/nucl	Aurora B/cyt	Aurora B/nucl
Grade				
G1	54.44±7.54	7.33±4.04	52.60±8.24	5.76±1.30
G2	63.18±7.88	9.96±3.56	52.36±8.11	13.93±2.52
G3	78.89±5.35	13.35±4.56	69.42±7.13	19.81±4.07
Stage				
pTa	57.30±5.31	8.13±2.81	53.26±5.63	10.85±1.77
pT1-2	80.58±5.51	14.44±4.26	59.52±8.22	17.62±3.89
MI				
≤5	55.59±7.49	7.21±3.89	47.88±8.26	6.46±1.29
>5	70.64±4.81	11.40±2.88	60.45±5.46	15.84±2.39

cyt: cytoplasmic; nucl: nuclear; MI: mitotic index.

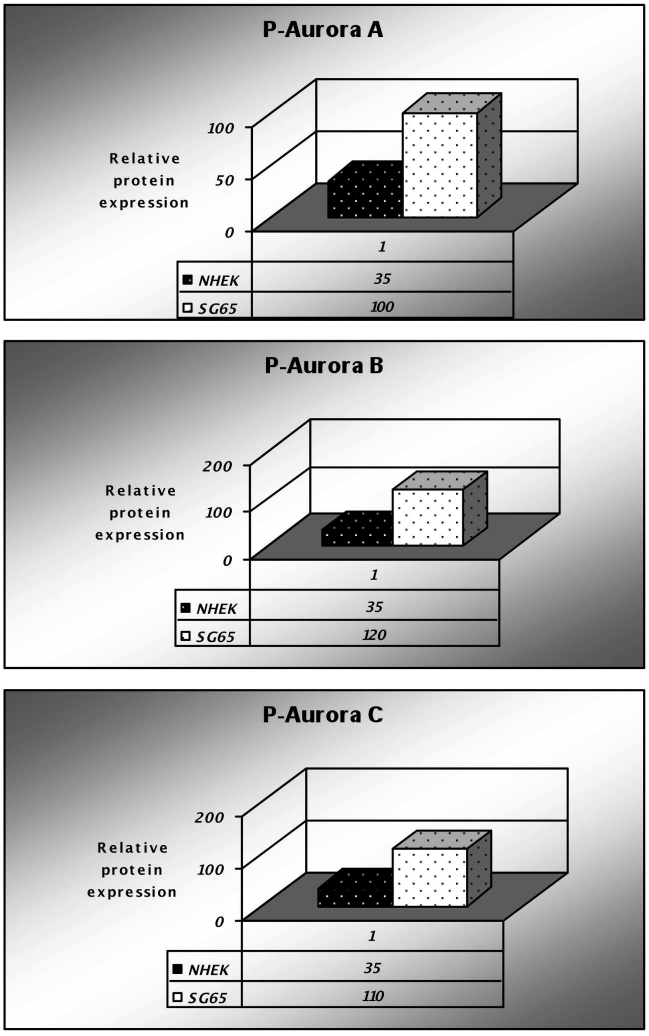

Fig. 1. Phospho Aurora A, B and C expression in aggressive bladder cancer cell line. Protein expression of cell lysates from NHEK and SG65 cells; Western blots were reacted with anti-phospho-Aurora A, B, C antibody, and the reaction was quantified by chemiluminescence and densitometric scanning. Data were normalised to ß-actin expression for each lane, for further details see material and methods.

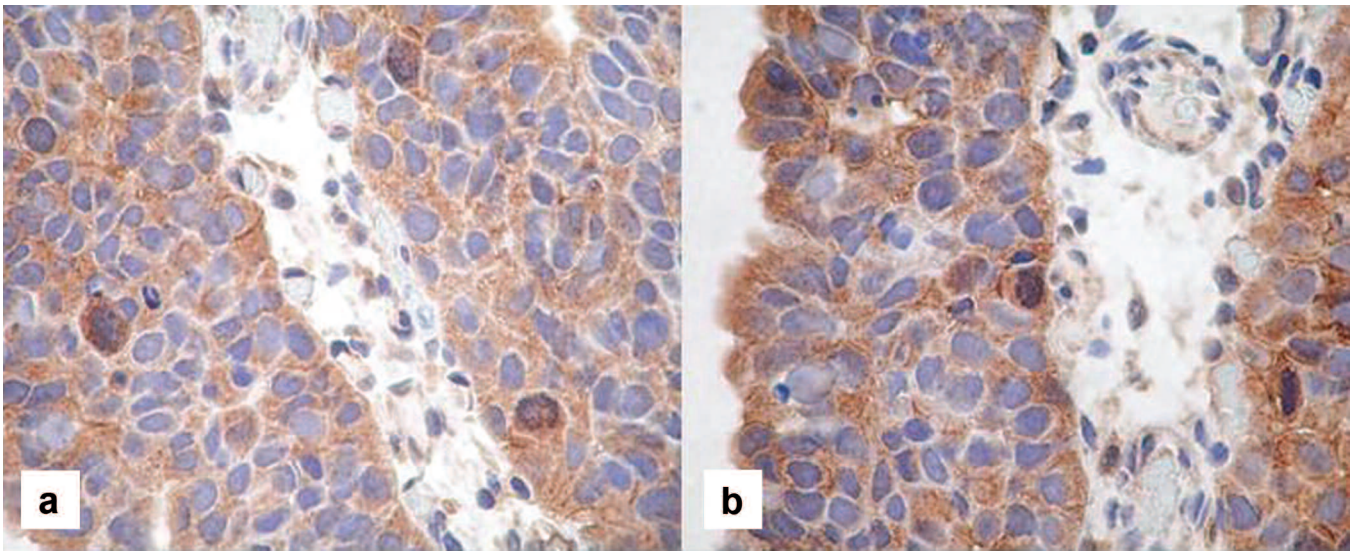

Fig. 2. Immunohistochemical staining of P-Aurora A in a G2pTa neoplasm: strong positivity was observed diffusely in cytoplasms **(a)** and in focal mitotic nuclei **(b)**. x 400

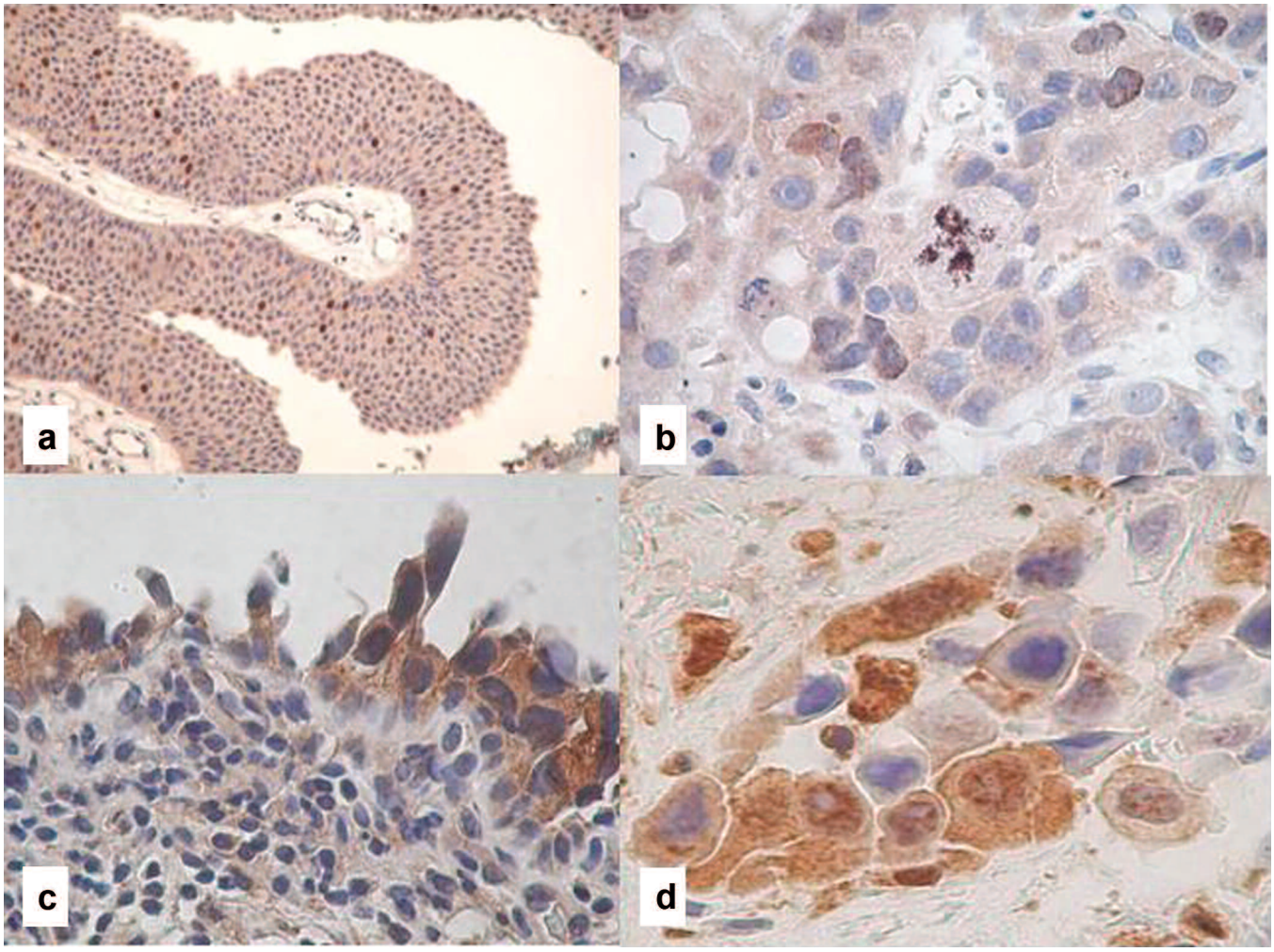

Fig. 3. Immunohistochemical expression of Aurora B. Diffuse cytoplasmic staining with scattered positive nuclei in a G2pT1 tumour **(a)**. Weak cytoplasmic positivity and focal nuclear staining in a G3pT2 tumour; a positive tetrapolar mitosis can be seen in the center **(b)**. Strong cytoplasmic positivity in a G3pT1 cancer with "clinging" aspects **(c,d)**. a, b, x 100; c, d, x 400

Aurora and Aurora B expression, both cytoplasmic and nuclear, was noted in tumours with higher grade and stage and it also correlated with mitotic activity (Figs. 4-6). Overexpression of P-Aurora A/cyt and of Aurora B/nucl was significantly more frequent in grade 3 tumours compared to grade 1 ($p<0.05$), and in grade 3 tumours compared to grade 2 and 1 ($p<0.01$), respectively. Moreover, a significant association between P-Aurora A/cyt overexpression and invasiveness was found ($p<0.01$). Greater levels of Aurora B/nucl were significantly associated with tumours carrying higher proliferation rates, as expressed by mitotic index >5 ($p<0.05$), regardless of tumour grade and stage.

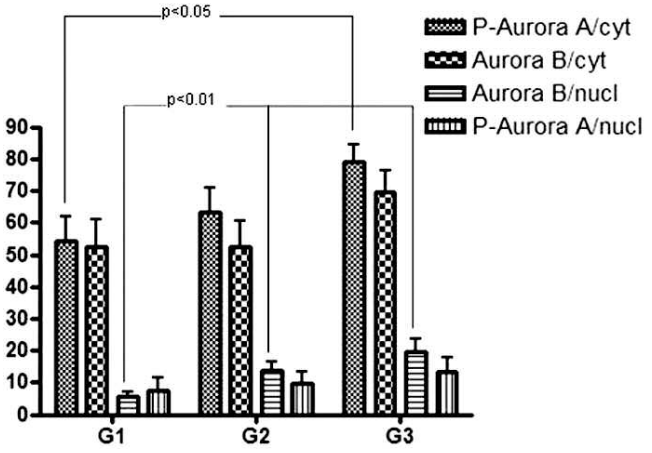

Fig. 4. Cytoplasmic and nuclear expression of p-Aurora A and Aurora B in urothelial neoplasms of different grade. P-value was estimated by the Fisher's exact test; cyt: cytoplasmic; nucl: nuclear.

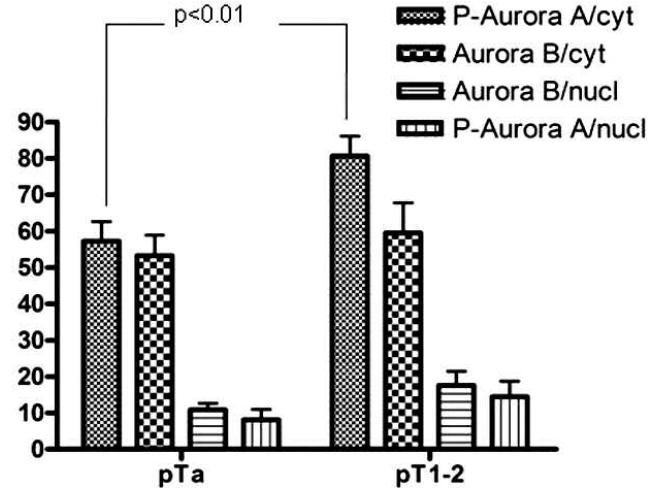

Fig. 5. Cytoplasmic and nuclear expression of p-Aurora A and Aurora B in non-invasive vs. invasive urothelial neoplasms. P-value was estimated by the Fisher's exact test; cyt: cytoplasmic; nucl: nuclear.

No significant association between immunohistochemical overexpression of any marker and other clinical factors (age, gender) was found.

Discussion

Since abnormal unregulated cellular proliferation is a peculiar feature of neoplastic diseases, cell division represents a crucial point in cancer development and progression. Mitosis is a multistep mechanism which is tightly orchestrated by the coordinated expression and function of several molecules, and a lack of proper mitotic regulation may lead to aneuploidy and chromosome aberrations. The Aurora family of serine/threonine kinases play a pivotal role in precise regulation of chromosome movement and organization, as well as formation of the mitotic spindle apparatus assembly (Glover et al., 1995; Bolanos-Garcia, 2005).

Several in vitro and in vivo studies so far addressed the association of Aurora kinases A and B with carcinogenesis, by demonstrating amplification and overexpression of such proteins in specific human tumours (Giet et al., 2005; Fu et al., 2007).

Aurora A is widely expressed in proliferating normal tissues. In vitro studies have identified Aurora A as a cancer-susceptibility gene (Ewart-Toland et al., 2003), and aberrations at different levels (amplification, transcriptional induction or post-translational stabilization) may lead to protein overexpression (Crane et al., 2004). Such overexpression was detected by Gritsko et al. as diffuse cytoplasmic in ovarian cancer tissues, thus resulting in abnormal phosphorylation of cytoplasmic proteins (Gritsko et al., 2003).

In the present consecutive series, distinct scores were assigned to nuclear and cytoplasmic staining of each marker, in order to get more precise information by the statistical analysis of discrete sets of data. Though Aurora A is known to accumulate on duplicate centrosomes until it degrades at G1, in tumour samples

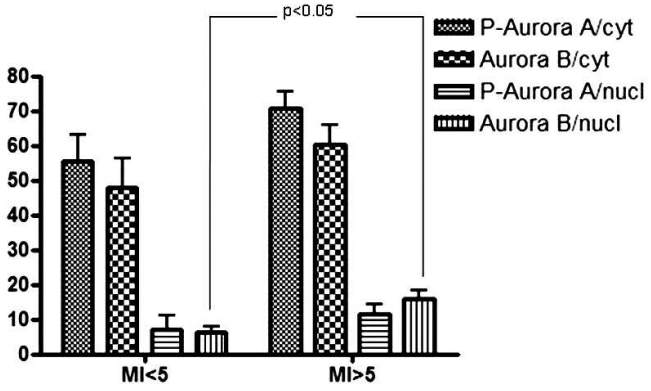

Fig. 6. Cytoplasmic and nuclear expression of p-Aurora A and Aurora B in relation with mitotic index. P-value was estimated by the Fisher's exact test; cyt: cytoplasmic; nucl: nuclear; MI: mitotic index.

its overexpression is usually found diffusely in the cytoplasm, rather than being restricted to certain areas (Dutertre et al., 2002). In keeping with such data, in our series cytoplasmic expression of P-Aurora A was by far more represented than the nuclear one, and it was diffuse in all positive cases; in particular, we found no perinuclear reinforcement of staining in the PUNLMP group, as highlighted by Compérat (2007) in his series of non-invasive bladder carcinomas.

Among all sets of data, in the current series P-Aurora A cytoplasmic pattern of immunostain provided the strongest statistical association with outcome-related clinicopathological parameters, i.e. tumour grade (G3 vs. G1, $p<0.05$), and stage (invasive vs. non invasive tumours, $p<0.01$). Such results confirm previous reports supporting the relation between Aurora A over-expression and/or amplification and bladder tumour aggressiveness (Sen et al., 2002; Compérat et al., 2007; Mhawech-Fauceglia et al., 2006). Interestingly, the only other study to examine the expression of both Aurora A and its phosphorylated form in superficial bladder tumours failed to find a statistical association with tumour grade and prognosis (Bruyere et al., 2008); such discrepancies may be due to the choice of different antibodies as well as to the different study setting, since only Ta neoplasms were included in the previous report.

A review of the literature shows that Aurora A may have close connections to well-known biomarkers of bladder cancer through different pathways, p53 being the most extensively sought so far. A more recent study by Liu et al showed that RASSF1A, a tumour-suppressor molecule whose aberrant methylation is frequently found in invasive high-grade bladder cancer, may also activate Aurora A in an indirect way (Liu et al., 2008).

As a member of the chromosomal passenger complex, along with the cancer-related protein survivin, Aurora B kinase locates to inner-centromeric chromatin and finally to the midzone through subsequent mitotic phases. Compared to Aurora A, its oncogenic potential has been far less investigated; current evidence suggests that Aurora B may act indirectly by strengthening other oncogenes (such as H-Ras) or by inducing genomic instability, rather than in a direct manner (Kanda et al., 2005). The few immunohistochemical studies on Aurora B expression in tumours report both cytoplasmic and nuclear pattern of immunostain (Lee et al., 2006), thus suggesting a possible intracellular translocation of this kinase which parallels the behaviour of survivin isoforms (De Maria et al., 2009). Furthermore, Aurora B kinase is enhanced by survivin binding (Bolton et al., 2002). The mechanism involved in the survivin regulation of Aurora B kinase activity and the exact interaction between the three main chromosomal passenger proteins is speculative at the present time. The discrepancy regarding survivin cytoplasmic versus nuclear location lies in the fact that survivin exists in two immunochemically distinct subcellular bladder cancer pools, possibly as a result of alternatively spliced transcripts (Fortugno et al., 2002). In our series, the nuclear expression of Aurora B was less represented than the cytoplasmic one, but it showed significant association with tumour proliferation activity as measured by the mitotic index ($p<0.01$). On the other hand, cytoplasmic staining of Aurora B displayed high statistical correlation with tumour grade (G3 vs. G2 vs. G1, $p<0.01$). These results seem to further support the pathogenetic role of Aurora B aberrations through each step of tumour progression from neoplasm to high-grade cancer, though a recent study addressing the expression of Aurora B in superficial bladder neoplasms found that high levels of such kinase, in presence of an aberrant G1 checkpoint, may have a protective role from tumour recurrence by impairment of proper mitotic functions (Bruyere et al., 2008).

Moreover, such highly-specific association between protein levels and each of the three grades of bladder neoplasms supports the reliability of current grading systems in assessing subtle differences among pathological entities.

To our knowledge, the present study is the first to investigate the association between expression of both the activated form of Aurora A and Aurora B kinases and the clinicopathological characteristics in a series of bladder neoplasms of different grade and stage. Since the search for molecular prognostic markers for bladder cancer is still a major clinical and therapeutic issue, our data provide basis for larger studies focused on the importance of detecting the expression of Aurora kinase proteins in clinical practice. This may be helpful in order to better differentiate among pathological entities on small biopsies, and even on cytological samples.

As for the therapeutic point of view, Aurora A expression is already known to play a prognostic role in human cancer, in that it showed an ability to induce cell resistance to tubulin-targeted agents, such as paclitaxel or nocodazole, by disruption of the spindle checkpoint activated by such therapies (Jiang et al., 2003). Moreover, the ongoing development of several small-molecule inhibitors represents a new promising direction for anticancer therapy, and a better clinical definition of such markers in each single human tumour is essential in order to gain real clinical benefit from these new targeted therapies.

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Accepted May 3, 2010