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Aurora B Expression Directly Correlates With Prostate Cancer Malignancy and Influence Prostate Cell Proliferation

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BACKGROUND. Chromosomal instability is one of the most common features of prostate cancer (PC), especially in advanced stages. Recent studies suggest that defects in mitotic checkpoints play a role in carcinogenesis. Lack of mitotic regulation induces aneuploidy in cancer cells acting thereafter as a driving force for malignant progression. Serine/threonine protein kinases of the Aurora genes family play an important throughout the entire cell cycle. In that Aurora B regulates chromosome segregation by ensuring the orientation of sister chromatids. As a consequence, the overexpression of Aurora B in diploid human cells NHDF induces the appearance of multinucleate cells.

METHODS. Archive samples of normal and neoplastic prostate tissue, and prostate derived cell lines were screened for the expression of Aurora B.

RESULTS. Immunohistochemical analysis showed increased nuclear expression of Aurora-B in high Gleason grade PCs respect to low and intermediate grade cases and in all cancers in respect to hyperplastic and normal glands. Furthermore, in the high Gleason grade anaplastic cancer tissues Aurora B expression was accompanied by the phosphorylation of the histone H3. In analogy to the in vivo situation, Aurora B was vigorously expressed in the androgen independent PC cell lines PC3 and DU145, while a very modest expression of the kinase was observed in the androgen sensitive LnCap cells and in the EPN cells, a line of epithelial cells derived from normal prostate tissue. In addition, in PC3 cells Aurora B expression is accompanied the by the phosphorylation of the histone H3. The block of Aurora B expression induced by an inhibitor of Aurora kinase activity significantly reduced the growth of prostate carcinoma cells, but not that of non-transformed EPN cells.

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CONCLUSIONS. Our data are the first demonstration of a role of Aurora B in PC progression. In addition, the observation that Aurora B specific inhibitors interfere with PC cell proliferation but not with that of non-transformed prostate epithelial cells suggest that Aurora B is a potential therapeutic target for PC. *Prostate 66: 326–333, 2006.* © 2005 Wiley-Liss, Inc.

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INTRODUCTION

Prostate cancer (PC) is the most common neoplasia in men and the second leading cause of death after cardiovascular diseases [1]. Because the complex structure of the prostate gland and the lack of appropriate model systems the mechanisms underlying initial development and progression of PC are still largely unknown. In addition, a part from PSA, reliable biochemical or genetic markers for early cancer detection or for prognostic use are not available at the moment and the diagnosis, and the disease management are complicated by the multifocal presentation and the phenotypic and genotypic heterogeneity of PC [2–4].

The molecular model of PC progression from initial normal dysplasia to terminal late-stage metastatic disease suggests the involvement of multiple genetic alterations. High-grade prostate intraepithelial neoplasia (HPIN) is the most likely precursor of PC [5].

Currently there is no available clinical, immunohistochemical, or morphologic criteria that are predictive of this progression. As a consequence it is very important to recognize early markers enabling us to discriminate between the pre-neoplastic lesion, which will progress into invasive cancer and those that will not progress. Chromosomal instability leading to the generation of numerical and structural changes has been implicated in the pre-neoplastic and neoplastic stages of PC. Unstable chromosome number in cancer cells is currently believed to act as a driving force during the malignant progression and aneuploidy is the most prevalent genomic alteration identified in solid tumors [6,7]. A critical step for the maintenance of genetic stability is the chromosome segregation, which requires a high coordination of cellular process [8,9]. Loss of the mitotic regulation is a possible cause of aneuploidy in human epithelial malignancy and it is thought to create an abnormal nuclear morphology in cancer cells. Serine/threonine protein kinases of the Aurora genes family play a regulatory role from G2 to cytokinesis, encompassing key cell cycle events such as centrosome duplication, chromosome bi-orientation, and segregation [10,11]. The family of the Aurora genes includes Aurora A/STK-15, Aurora B/AIM-1, and Aurara C/AIK3. Aurora A is overexpressed in several solid tumors, and NIH 3T3 fibroblasts overexpressing Aurora A gene show an increase in centrosome number and aneuplody leading to neoplastic transformation [12–14].

Aurora B is a chromosome passenger protein, localized on the centromeres from prophase through the metaphase-anaphase transition [10,14–19]. Another chromosome passenger protein is INCENP, which tightly associates with Aurora B, probably regulating its activity. Survivin, a conserved inhibitor of apoptosis (IAP) like protein is a third member of the Aurora B complex [8,9]. Aurora B has been described to phosphorylate histone H3 at ser-10 and phosphorylation of histone H3 is required for proper chromosome dynamics during mitosis [14–19]. It has been also demonstrated that Aurora B overexpression in diploid human cells NHDF induces multinuclearity. Furthermore, Aurora B transfected cells have an unstable chromosome number and show an aggressive phenotype in vivo [18,19].

Since Aurora B plays a crucial role for chromosome dynamics, and aneuploidy is a common feature of prostate carcinomas, we analyzed Aurora B expression in archive prostate carcinoma tissue and in human prostate cell lines, originating from normal and neoplastic prostate tissue. All of prostate carcinoma cell lines showed increased Aurora B levels compared to the non-transformed cell line. Immunohistochemical studies performed on archive samples showed that Aurora B was detectable in normal prostate tissue and that its expression progressively increased with increasing of the Gleason Score, with a strong signal in anaplastic prostate carcinomas. The block of Aurora B kinase B activity by means of specific kinase inhibitors induced growth inhibition in human prostate anaplastic carcinoma cells but not in non-transformed prostate epithelial cells.

MATERIALS AND METHODS

Cell Culture

The two androgen-independent human PC cell lines PC3 [20] and DU145 [21] and the androgen-sensitive cell line, LnCap [22] used in these studies were grown in DMEM supplemented with 10% fetal bovine serum. EPN cells, a line of non-transformed epithelial cells derived by human normal prostate tissue spontaneously adapted to grow in culture, were routinely

cultured in Keratynocyte serum free medium (KSFM) supplemented with 3% fetal bovine serum (FBS) [23]. Growth curves were performed as previously described [23]. As for evaluation of the effect of the kinase inhibitor on cell proliferation the following method was applied. DU145, LnCap, PC3 cells were plated at a concentration of 1×10^3 cell/well in a 96-well plate and after 24 hr the cells were treated with 5, 10, 15 μM of the inhibitor. The quinazolin derivative: *N*-[4-(6,7-dimethoxy-quinazolin-4-ylamino)-phenyl]-benzamide [24] was dissolved in DMSO at a concentration of 10 mM. Cell proliferation was evaluated after 24 and 48 hr by using the Cell Titer 96® Aqueous cell proliferation assay (Promega, Milan, Italy). The values reported represent the mean ± SD of three independent samples per each experimental point.

FACS

Cytofluorimetric estimation of cell proliferation was performed as follows: the plates were washed in cold PBS and trypsin was added. Cells were collected, washed again in PBS and fixed in cold PBS, 70% ethanol for 30 min. Ethanol was removed by two PBS washes, and cells were incubated in PBS, 50 μg/ml propidium iodide, 10 μg/ml ribonuclease A, and deoxyribonuclease-free overnight at 4°C. Cells were then analyzed by flow cytometry using a FACScan (Becton Dickinson, Mountain View, CA). G_0–G_1, S, and G_2–M cell cycle phases were gated and the corresponding cells were counted.

Histology

Archival formalin-fixed, paraffin-embedded prostatectomy tissue was acquired from the files of the Pathology Section of the Biomorphological and Functional Sciences Department, University of Naples Federico II, Naples, Italy.

A total of 35 cases of PC were retrieved and, for each case, a paraffin block from the most representative area of the tumor was selected, as well as a block selected from different regions of the same prostate containing normal and/or hyperplastic prostatic glands. Four micrometer-thick serial sections were then cut from each block, and a hematoxylin and eosin section of each tumor was re-examined to confirm the original diagnosis and staging of PC. The Gleason sum scores for the cases ranged from 4 to 9.

Immunohistochemistry

Serial sections of each case were de-waxed, rehydrated through decreasing alcohols and treated with 3% hydrogen peroxide for 5 min to inactivate endogenous peroxidases, and then washed in distilled water. The slides were then pre-incubated in a microwave oven three times for 5 min in 10 mM, 6 pH buffer citrate. After quenching of endogenous peroxidase, antigen retrieval was achieved by boiling sections at 120°C at 3.4 atm for 10 min in 1% citrate buffer; the sections were then blocked for 2 hr at room temperature with 1.5% blocking serum (DAKO). Incubation with the primary antibody was carried out overnight, in a moist chamber, with the anti AIM-1 (Aurora B, 1:1,000, #611082,BD Transduction Laboratories, San Diego, CA). Finally, binding of the primary antibody was detected by the conventional streptavidin-biotin linked horseradish peroxidase (LSAB-HRP) procedure, using 3,3′-diaminobenzidine (DAB) as chromogen [25]. Negative controls were performed on serial sections of the same tissues, substituting the primary antibody with non-immune serum.

Slight nuclear counterstaining was performed with Harris' hematoxylin; slides were then coverslipped using a synthetic mounting medium.

The results of the immunohistochemical staining were evaluated separately by two observers. Only cells with a definite brown staining were judged as positive.

The immunohistochemical expression was quantified semi-quantitatively, using a 40× objective and expressed as percentage of positive nuclei. Then, tumors and respective normal and/or hyperplastic glandular tissue were assigned to one of the following arbitrary categories: (1) from 0% to <5% of positive cells; (2) from 5% <10%; (3) from 10% to 25%; (4) >25% of cells.

The data were analyzed utilizing the one-way Analysis of Variance (ANOVA) and Student-Newman–Keuls Multiple Comparisons Test (SPSS statistical software, version 6.1 for Windows. For the categorical parameters the Chi-square test was used; the non-parametric Mann–Whitney *U*-test was utilized for continuous variables). Only values of $P < 0.05$ were considered as significant.

Protein Extraction and Western Blot Analysis

Prostate samples or cells were homogenized directly into lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1%Triton-X-100, 1 mM phenylmethylsulfonyl fluoride, 1 μg aprotinin, 0.5 mM sodium orthovanadate, 20 mM sodium pyrophosphate). The lysates were clarified by centrifugation at 14,000× 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 condition. After

electrophoresis, proteins were transferred to nitrocellulose membranes (Immobilon Millipore Corporation); complete transfer was assessed using prestained protein standards (Bio-Rad, Hercules, CA). After blocking with TBS-BSA (25mM Tris, pH 7.4, 200 mM NaCl, 5% bovine serum albumin), the membrane was incubated with the primary antibody against: (1) Aurora B (1:400; # 611082, BD Transduction Laboratories); (2) Phospho H3 (ser 10) (1:250, # 06-570, Upstate, Lake Placid, NY); (3) β-Tubulin (1:500, # T-4026, SIGMA Chemical Corporation, St. Louis, MO) for 1 hr (at room temperature). Membranes were then incubated with the horseradish peroxidase-conjugated secondary antibody (1:2,000) for 45 min (at room temperature) and the reaction was detected with an ECL system (Amersham, Life Science, Chiltern Hills, UK).

RESULTS

Aurora B Expression in ProstateTissue

A total of 35 cases of archive prostate tissue comprising normal prostate and neoplastic tissues scoring Gleason 4 to 7/8 were retrieved and the original histological diagnosis and grading of PC was confirmed for all the cases examined. Tissues were stained with anti Aurora B antibodies. Results showed increased nuclear expression of Aurora-B in high-score PCs respect to low and intermediate score cases ($P < 0.05$) and in all cancers respect to hyperplastic ($P < 0.05$) and normal ($P < 0.01$) glands (Table I, Fig. 1a–c).

Four cases of PC showed a diffuse, strong cytoplasmic staining, in addition or instead of the nuclear staining. In two cases of benign hyperplasia a cytoplasmic localization of Aurora B immunostaining was observed in isolate epithelial cells. Dynamic exchange from cytoplasm and centrosome has been already reported for other members of the Aurora gene family [26,27]. In normal prostate tissue, few cells positive for Aurora B staining were observed (Fig. 1a). The expression of Aurora B was confined to the epithelial cells and no positive cells could be observed within the stromal part of the gland. Similarly to that of Aurora B the expression of Ki-67 increased in tumors tissues in respect to normal or hyperplastic areas (data not shown).

Western Blot analysis of total protein extracted from the frozen prostate tissue comprising normal prostate and neoplastic tissues scoring Gleason 4 to 7/8 confirmed the morphological results. A band of 41 kDa was observed in all the samples tested. Aurora B expression dramatically increased with the Gleason score, being extremely high in undifferentiated tumors (Fig. 2a). Aurora B has been described to phosphorylate histone H3 at Ser-10, therefore to investigate the biological activity of the kinase in the prostate tissue, the same samples were immunoblotted with anti-phospho-Ser-10 H3 histone antibodies. As shown in Figure 2b, an increased phosphorylation of the H3 histone was observed in the higher Gleason grade tissues. β-Tubulin was used to assess the equal amounts of protein (Fig. 2c).

Aurora B Expression in Human Prostate Cell Lines

The expression of Aurora B was investigated in prostate cell lines derived from normal and neoplastic tissues by Western blot. As shown in Figure 3a Aurora B was expressed in the androgen independent PC3 cell line and at a lesser extent in the DU145 cell lines while a much weaker expression of the kinase was observed the androgen sensitive LnCap cells. In the EPN cells, a line of non-transformed androgen sensitive epithelial cells the expression of Aurora B was consistently weaker then that of the tumor cell lines. In addition, in PC3 and in DU145 cell lines Aurora B expression is associated to an increase in the phosphorylation of histone H3 (Fig. 3b). β-Tubulin was used to assess the equal amounts of protein (Fig. 3c).

TABLE I. Immunohistoclinical expression and topographical localization of AURORA B in 35 cases of prostate cancer.

Cases	Gleason's score	Aurora B score	Normal prostaic glands	Benign hyperplastic glands
N = 14	≤4	2 (n = 6, N*)	1 (N = 7)	1 (N = 3)
		3 (n = 6, N)	2 (N = 7)	2 (N = 11)
		4 (n = 2, N)		
N = 12	>4 to ≤7	3 (n = 5, N)	1 (N = 2)	2 (N = 12)
		4 (n = 7, N)	2 (N = 10)	
N = 9	>7	4 (n = 7, 5N, 2N+C**)	2 (N = 9)	2 (N = 7) 3 (N = 2)
		4 (n = 2, C)		

*Nuclear.
**Cytoplasmic localization of Aurora B.

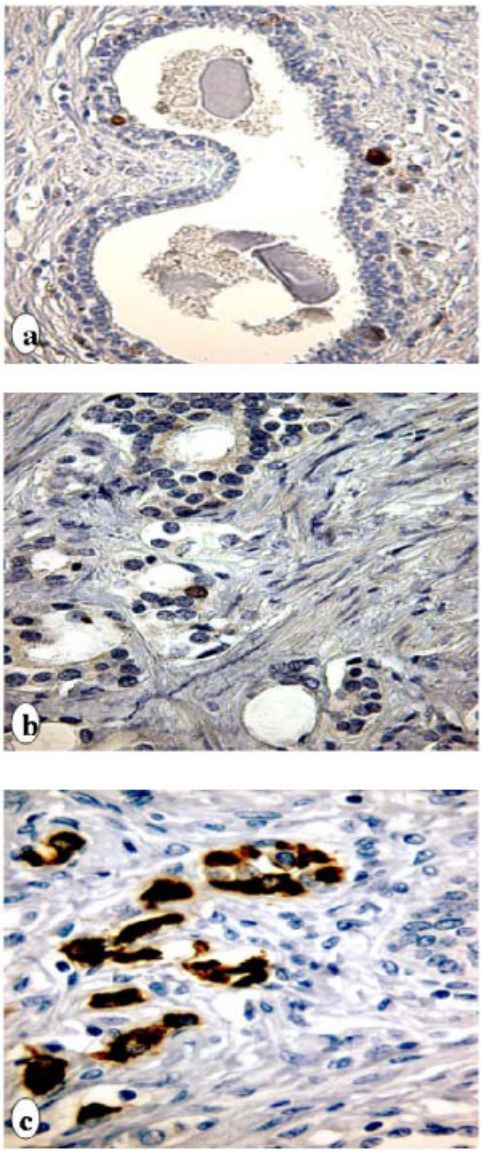

Fig. 1. Aurora B expression in human prostate tissues. Localization of the Aurora B protein in human prostate tissue by immunocytochemistry; **(a)** paraffin embedded section of normal prostate tissue. Expression of Aurora B protein was not observed; **(b)** section of intermediate Gleason grade prostate carcinoma, showing some nuclear immunoreactivity for Aurora B protein; **(c)** section of anaplastic prostate carcinoma, showing extensive nuclear immunoreactivity for Aurora B protein (400×). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Aurora B Kinase Inhibitor and Prostate Cell Proliferation

A small molecule similar to the quinazolin derivative: *N*-[4-(6,7-dimethoxy-quinazolin-4-ylamino)-phenyl]-benzamide has been shown to inhibit the Aurora B kinase activity [24,28]. To evaluate the effects of Aurora kinase inhibitor on prostate cell proliferation, PC3, DU145, LnCap, and EPN cells were treated with 5, 10, and 15 μM, and cell proliferation was evaluated after 24 and 48 hr. The Aurora kinase inhibitor significantly reduced the proliferation of PC cells, while had no effects on the non-transformed EPN cells, which expressed only small concentrations of Aurora B

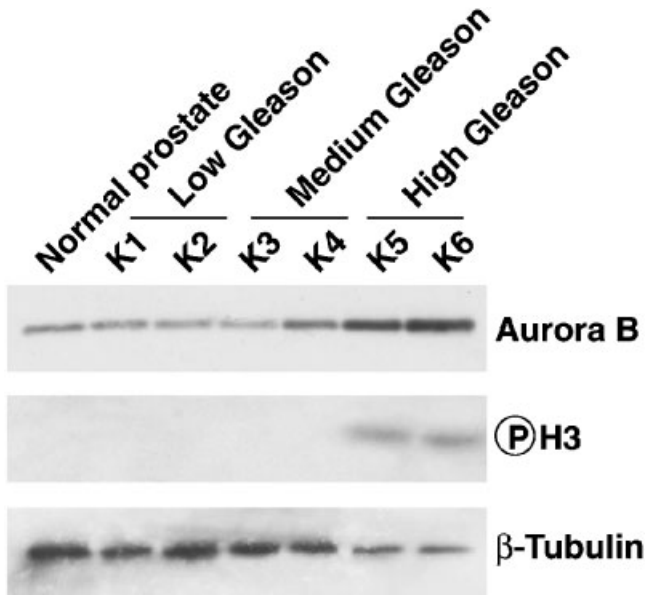

Fig. 2. Western blot analysis of Aurora B expression in prostate human tissues; **(a)** the expression of Aurora B protein increases with increasing Gleason score being maximal in anaplastic carcinomas; **(b)** in addition in the samples corresponding to high grade carcinoms it present a band corresponding to the phosphorylated hystone H3; **(c)** β-Tubulin was used to assess the equal amounts of protein.

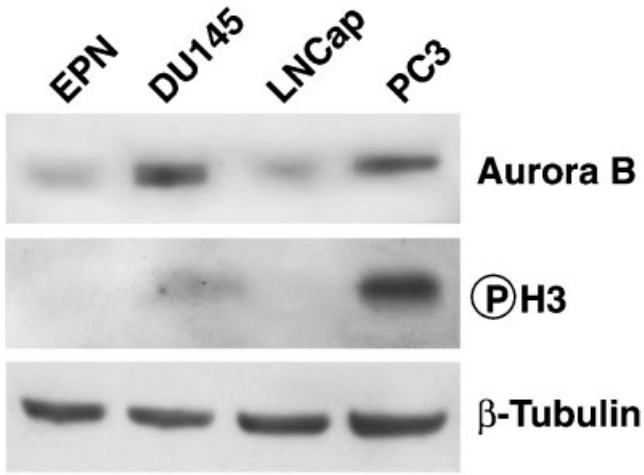

Fig. 3. Western blot analysis of Aurora B expression in human prostate cell lines; **(a)** the level of expression increased from normal differentiated androgen dependent to non-differentiated androgen independent cell lines. Aurora B was highly expressed in the PC3; at a lesser extent in the DUI45 cells, and it was basically absent in LnCap and in the EPN cells; **(b)** in the PC3 Aurora B mediated the phosphorylation of H3 hystone; **(c)** β-Tubulin was used to assess the equal amounts of protein.

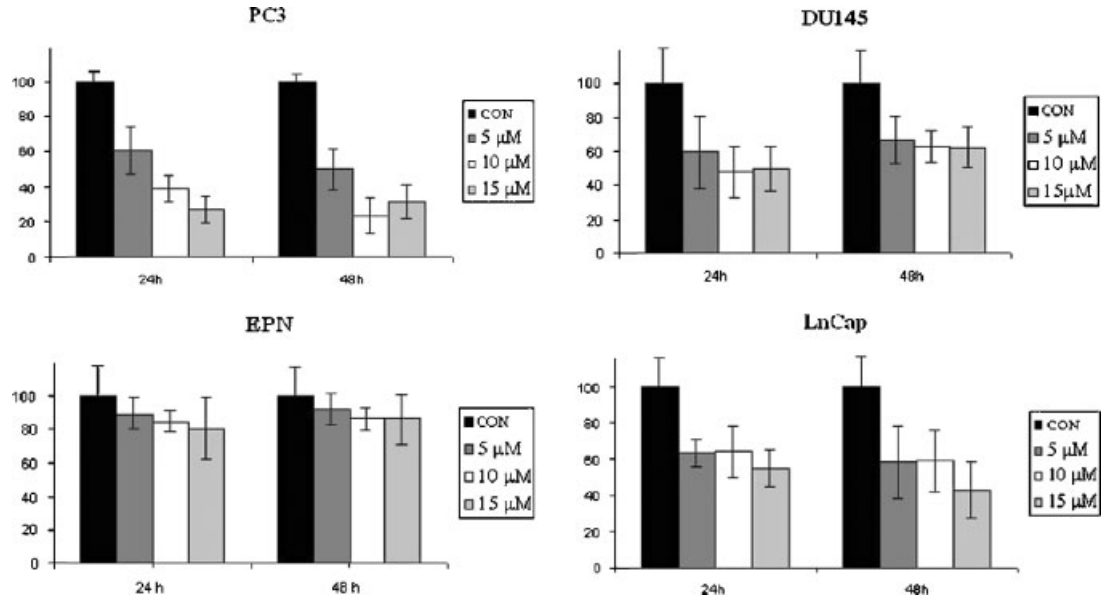

Fig. 4. Aurora kinase inhibitor reduced the growth of prostate tumor cells PC3, DU 145, and LnCap but not that of normal prostate epithelial cells, EPN.

kinase (Fig. 4). As expected FACS analysis showed that the kinase inhibitor blocked the cell cycle of PC3, DU145, and LnCap cells at the G_2/M phase.

DISCUSSION

In the present study we have evaluated the expression of Aurora B in prostate cell lines and human prostate tumors. The immunohistochemical analysis of neoplastic or non-neoplastic prostate tissues showed that the expression of Aurora B directly correlates with increase in the Gleason grade of the PC being abundant in undifferentiated carcinomas. Conversely, lower expression was observed in normal tissues and in low-grade prostate carcinomas. This observation suggests that during tumor progression, in particular during the transition from differentiated prostate carcinomas to undifferentiated prostate carcinomas, Aurora B overexpression takes place and Aurora B overexpression might confer a growth advantage to the neoplastic cells [29–33].

By using a selective Aurora B kinase inhibitor we demonstrated that the block of Aurora B activity dramatically reduces the proliferation of prostate carcinoma cells [24,28]. It is important to note that the growth of EPN cells, a line originated from normal epithelial prostate tissue, which express low levels of Aurora B, was not affected by the Aurora B inhibitor.

In LnCap cells the expression of Aurora B is weaker than that in PC3 and Du145. It is important to note that LnCap cells, although derived from a lymph node of an aggressive PC, are among the PC derived cell lines, the less aggressive and tumorigenic. Moreover, LnCap cells retain prostate differentiated markers including the sensitivity to androgen stimulation, also suggesting a less aggressive phenotype. In addition, the proliferation rate is much lower than that of PC3 and Du145 [22], therefore the expression of Aurora B correlates with the proliferative rate and the aggressiveness of the different cell lines.

Alterations of the mechanisms responsible for maintaining the chromosome balance are common in neoplastic cells [15,16]. Disruption of mitotic checkpoints induces unstable chromosome number or aneuploidy, which is thought to create abnormal nuclear morphology in cancer cells and contribute to genetic instability. Alterations in chromosome number have been described as a common feature of almost all solid tumors. Aneuploidy has been observed also in PC. Interestingly, studies using loss of heterozygosity analyses, CGH2 and fluorescent in situ hybridization revealed few recurrent chromosomal aberrations in early PCs [34–36]. Low-stage primary tumors tend to be near diploid with few clonal numerical and/or structural aberrations. In contrast, high-stage, advanced primary, or metastatic prostate tumors tend to be aneuploid with a high degree of chromosomal aberrations [2,8]. Moreover several chromosomal abnormalities have been reported and summarized in the expert reviews published on http://www.expert reviews.org in 2003 [37].

Although chromosomal instability is a key mechanism in the process of malignant transformation in human epithelial tissues, yet the molecular mechanisms responsible for chromosome destabilization during carcinogenesis are largely unknown [38–41].

Centrosome defects have been invoked to contribute to genomic instability during PC progression. Centrosomes are structurally and numerically abnormal in the vast majority of metastatic and invasive prostate carcinomas. The extent of centrosome abnormalities in invasive prostate carcinoma correlates with the Gleason grade [42].

Two recent set of data reported the potential role of STK15 (Aurora-A) a serine/threonine kinase found in centrosomes and involved in mitotic chromosomal segregation in PC. Overexpression of Aurora-A is present in some normal and in the majority of high-grade PIN lesions and is an early event leading to the genetic instability in prostate carcinogenesis [43,44].

Thus, dysfunction in mitotic checkpoints represent a primary cause of genomic instability and as a consequence of accumulation of structural and numeral chromosomal abnormalities present in tumor cells. Aurora B assuring the correct orientation of sister chromatides plays a crucial role in mitosis. A significant overexpression of Aurora B has been observed in human cancer cell lines, and in colorectal tumors a correlation between Aurora B expression levels and Duke's grade in colorectal tumors has been described [33]. Furthermore, it has been observed that the forced expression of Aurora B produced aneuploid cells with a malignant and aggressive phenotype, indicating that Aurora B is relevant in aneuploidy development during carcinogenesis [18,19]. Recently, we have demonstrated that high expression levels of Aurora B are a consistent feature of human seminomas and in thyroid anaplastic carcinomas a high expression of Aurora B was observed [45–47]. In these previous studies, the expression of Ki-67 was analyzed showing an increase in tumors tissues in respect to normal or hyperplastic areas similar to Aurora B. In agreement with our previous results a highly statistical correlation between expression Aurora B and that of Ki-67 with a percentage of Aurora-B positive cells lower than that of the Ki-67-positive cells (data not shown) was observed.

CONCLUSIONS

Although large efforts have been put in the research of appropriate therapy of PC the appearance in the population of the tumor of androgen insensitive cells vanishes the good result obtained by the hormonal therapy. The observation that in PC tissue Aurora B gene expression levels directly correlates to the stage of malignant progression, that the block of Aurora B expression or activity via an Aurora B inhibitor, reduces the proliferation of anaplastic prostatic carcinoma cells but not that of epithelial normal cells could contribute to the development of novel therapeutic strategies. The implications of these findings include Aurora B assessment as an useful biomarker in the gray zone for monitoring disease in prevention strategies and for improving early diagnosis.

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