Int. J. Cancer: 119, 275–282 (2006)
© 2006 Wiley-Liss, Inc.

Expression of Aurora kinases in human thyroid carcinoma cell lines and tissues

Salvatore Ulisse[1], Jean-Guy Delcros[2], Enke Baldini[1], Matteo Toller[3], Francesco Curcio[3], Laura Giacomelli[4], Claude Prigent[5], Francesco S. Ambesi-Impiombato[3], Massimino D'Armiento[1] and Yannick Arlot-Bonnemains[2]*

[1]*Department of Experimental Medicine and Pathology, University of Rome "La Sapienza", Rome, Italy*
[2]*Groupe Cycle Cellulaire et Pharmacologie, CNRS-UMR 6061 "Génétique et Développement", IFR 140 G.F.A.S., Faculté de Médecine, Université Rennes 1, Rennes, France*
[3]*Department of Pathology and Experimental Medicine and Clinic, University of Udine, Italy*
[4]*Department of Surgical Sciences, University of Rome "La Sapienza", Rome, Italy*
[5]*Groupe Proteines kinases mitotiques et oncogénèse- CNRS-UMR CNRS 6061 "Génétique et Développement," IFR 140 G.F.A.S., Faculté de Médecine, Unizersité Rennes 1, F-35043 Rennes Cédex, France*

The Aurora kinases are involved in the regulation of cell cycle progression, and alterations in their expression have been shown to associate with cell malignant transformation. In the present study, we demonstrated that human thyrocytes express all 3 Aurora kinases (A, B and C) at both protein and mRNA level and this expression is cell cycle-regulated. An increase in the protein level of the 3 kinases was found, with respect to normal human thyrocytes (HTU5), in the human cell lines derived from follicular (FTC-133), papillary (B-CPAP) and anaplastic (8305C) thyroid carcinomas, but not in cells derived from a follicular adenoma (HTU42). These observations were mirrored in RT-PCR experiments for Aurora-A and B. In contrast, Aurora-C mRNA levels were not significantly different among the different cell types analyzed, suggesting that posttranscriptional mechanism(s) modulate its expression. The expression at the protein level of all 3 Aurora kinases was significantly higher in 3 thyroid papillary carcinomas with respect to normal matched tissues obtained from the same patients. Similar modifications, at the mRNA level, could be observed in 7 papillary carcinoma tissues for Aurora-A and B, but not for Aurora-C. In conclusion, we demonstrated that normal human thyrocytes express all 3 members of the Aurora kinase family, and their expression is amplified in malignant thyroid cell lines and tissues. These results suggest that the Aurora kinases may play a relevant role in malignant thyroid cancers, and may represent a putative therapeutic target for thyroid neoplasms.
© 2006 Wiley-Liss, Inc.

Key words: Aurora kinases; thyroid; human cancer

Neoplasms derived from the follicular thyroid cell represent the most common endocrine malignancy accounting for roughly 1% of all new malignant diseases and about 0.4% of deaths related to cancer.[1] The large majority of follicular thyroid cancers are represented by the differentiated papillary and follicular thyroid carcinomas, which, following dedifferentiation, are thought to give rise to the highly aggressive and fatal anaplastic thyroid carcinomas.[2,3] Although derived from the same cell type, the different thyroid neoplasms show specific histological features, biological behavior and degree of differentiation, as a consequence of different genetic alterations.[4–8]

As other types of solid neoplasms, thyroid follicular cancer cells are often characterized by chromosomal instability and aneuploidy.[8–13] The serine/threonine kinase Aurora family has been shown to play a key role in the regulation of multiple aspects of chromosome segregation and cytokinesis.[14,15] In human, the Aurora family includes 3 members: Aurora-A, -B and -C, which share a 67–76% aminoacid sequence identity in their catalytic domains, and little similarities are present in their N-terminus, providing the molecular basis for specific interactions with different substrates and for the different subcellular localizations.[14,15] Their expression and activity is tightly regulated during the cell cycle; in particular, the Aurora proteins start to accumulate at the end of the S phase and their expression is maximal in the G2/M phase to be reduced at the beginning of the G1 phase.[16] Each of the kinases display a specific localization. Aurora-A is associated with centrosomes and is involved in their positioning, recruitment of components at the forming mitotic spindle and microtubules stability.[17] Its rapid degradation at the end of mitosis by the ubiquitin–proteasome pathway has been shown to be required for the cell entry into a new cell cycle.[18] Aurora-B is a chromosomal passenger protein, which associates with chromatin at the beginning of mitosis and, as chromosomes condensation occurs, it forms a complex with other chromosomal passenger proteins, such as INCENP and survivin, leading to the phosphorylation of histone H3.[15,19,20] Moreover, during the transition from anaphase to telophase, Aurora-B has been demonstrated to play a major role in the mitotic spindle dynamics and cleavage furrow, and it can be observed in the midbody of cytokinetic cells.[19,21] Recently, also the Aurora kinase C has been demonstrated to be a chromosomal passenger protein, colocalized and able to form a complex with Aurora-B, INCENP and survivin in mitotic cells.[22]

The genes encoding the 3 Aurora kinases map into regions that are affected by chromosomal abnormalities in different cancer types, and their overexpression has been detected in several tumor cell lines.[23] Aurora-A gene lies within the human chromosome region 20q13, and it is amplified in many forms of cancer such as bladder, ovarian and, at high frequency, in colorectal tumors.[23–27] In addition, most of the primary invasive mammary carcinomas analyzed for Aurora-A immunoreactivity were found to be strongly positive.[28] A significant overexpression of Aurora-B has been described in human cancer cell lines, and a correlation between Aurora-B expression levels and Duke's grade in colorectal tumors has been described.[29,30]

Concerning the thyroid tissue, only Aurora-B expression has been recently investigated in a single report and showed to be present in normal tissue and upregulated in undifferentiated thyroid cancer cells and tissues.[31] In our study, we analyzed the expression and cellular localization of all the 3 Aurora kinases in human cell lines derived from normal thyrocytes and different histotypes of thyroid tumors, including a benign follicular adenoma. We further extended the analysis of their expression to normal and papillary carcinoma thyroid tissues obtained following thyroidectomy.

Material and methods

Cell lines and materials

The cell lines 8305C[32] and B-CPAP[33] were obtained from German Collection of Microorganisms and Cell Cultures (DMSZ, Germany), while FTC-133 cells[34] were obtained from Interlab

Grant sponsors: Agenzia Spaziale Italiana (ASI); Ministero dell'Istruzione; dell'Università e della Ricerca (PRIN 2004); "Ligue Nationale contre le cancer"; and Conseil Regional de Bretagne.
***Correspondence to:** Cycle cellulaire et Pharmacologie, CNRS -UMR 6061 Génétique et Développement, Université de Rennes 1, 2 Avenue du Pr Léon Bernard, CS 34317, 35043 Rennes Cedex, France.
Fax: 33-223234478. E-mail: yannick.arlot@univ-rennes1.fr
Received 7 October 2005; Accepted 21 December 2005
DOI 10.1002/ijc.21842
Published online 13 February 2006 in Wiley InterScience (www.interscience.wiley.com).

Publication of the International Union Against Cancer
uicc global cancer control

TABLE I – PRIMER SEQUENCES, EXON POSITIONS, SIZE OF AMPLIFIED PRODUCTS, ANNEALING TEMPERATURES AND NUMBER OF CYCLES USED IN THE PCR REACTION FOR THE DIFFERENT MEMBERS OF THE AURORA KINASE FAMILY

Gene	Primers	Exon	Size (bp)	T_{ann} (°C)	No of cycles
Aurora-A	Forward 5′-CTGCATTTCAGGACCTGTTAAGG-3′	1	150	60	27
	Reverse 5′-AACGCGCTGGGAAGAATTT-3′	2			
Aurora-B	Forward 5′-CGACATCTTAACGCGGCAC-3′	3–4	50	60	30
	Reverse 5′-GGACGCCCAATCTCAAAGTC-3′	4			
Aurora-C	Forward 5′- TATAACTATTTCCATGATGCACGCC-3′	4	167	60	35
	Reverse 5′-ACTTTCTTGTCATGGCAGTAGGTC-3′	5			
β_2 Micro, globulin	Forward 5′-TGACTTTGTCACAGCCCAAGATA-3′	2	75	60	20
	Reverse 5′-CGGCATCTTCAAACCTCCA-3′	3–4			

Cell Line Collection (ICLC, Italy). Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 medium, HAM'S nutrient mixture F-12, phosphate buffered saline (PBS), foetal bovine serum (FBS), trypsin, EDTA, L-glutamine 100X (200 mM) and penicillin/streptomycin solution 100X were obtained from EuroClone (Paignton-Devon, UK). RNAzol™ was provided from Biotech Italia (Roma, Italy). Oligo$(dT)_{12\text{-}18}$ primer, dNTP mix and M-MLV reverse transcriptase were obtained from Invitrogen (Carlsbad, CA). HotMaster™ Taq DNA polymerase and Perfectprep® Gel Cleanup Kit were obtained from Eppendorf (Hamburg, Germany). All primers were from Eurogentec (Seraing, Belgium) and 100 bp DNA ladder from New England BioLabs (Beverly, MA). Sodium deoxycholate, aprotinin, leupeptin, 4-(2-amino-ethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), sodium orthovanadate, sodium pyrophosphate and the antiactin antibody were all obtained from Sigma Chemical (St. Louis, MO). Nonidet P-40 (NP-40) was from Calbiochem (La Jolla, CA). Protein Standard™, Bradford protein assay kit and electrophoresis reagents were obtained from Bio-Rad Laboratories (Hercules, CA). The monoclonal antibody against Aurora-A was produced in the laboratory. The polyclonal antibody against Aurora B and Aurora-C were obtained from Abcam (Cambridge, UK) and Abgent (San Diego, CA), respectively. The anti-mouse and anti-rabbit horseradish peroxidase conjugated secondary antibodies were from Jackson Immuno-Research Laboratories (Baltimore, MD).

Cell cultures and human thyroid tissues

The 8305C and B-CPAP cells were cultured in RPMI 1640 medium with 5% FBS, while the FTC-133 line in DMEM mixed to HAM'S/F-12 in ratio 1:1, supplemented with 5% FBS and 2 mM L-glutamine. One hundred U/l penicillin and 100 µg/ml streptomycin were all added to the media. The normal strain of human thyroid cells (HTU5) and the follicular adenoma derived cell line (HTU42) were grown, as previously described.[35,36] The cells were maintained in continuous monolayer cultures at 37°C and 5% CO_2, expanded up to 70–80% confluence and then employed for the experiments, as described below. To investigate the effects of serum on normal human thyrocyte proliferation and Aurora kinases expression, cells have been cultured for 4 days in the presence of 5% FBS or 0.3% FBS. Following 4 days in medium containing low serum concentration, the same cells have been treated for 24 hr in medium containing 5% FBS. The cells have been then processed for FACS analysis or to prepare total RNA and cell protein extracts.

Fragments of normal and tumoral thyroid tissues were obtained from surgical specimens of 7 female patients (age ranging 36–76 years) affected by the classical variant of papillary thyroid carcinomas. Tissue samples were immediately frozen in liquid nitrogen, stored at −80°C and then used for the preparation of total RNA or protein extracts, as described below.

RNA isolation and analysis

Total cellular RNA was extracted from the different cell lines by the acid guanidinium thiocyanate-phenol-chloroform method of Chomczynsky and Sacchi, according to the standard procedure.[37] The same protocol was also used to obtain total RNA from normal and tumoral human thyroid tissues, following homogenization of the samples with guanidinium thiocyanate for 30 sec, using Ultra-Turrax. Five microgram of total RNA were reverse-transcribed using oligo-dT primers and M-MLV reverse transcriptase. The obtained cDNAs were used as templates for the subsequent PCR amplifications of the Aurora kinases A, B, C and human β_2-microglobulin, as internal control, using specific primers described in Table I. Amplifications were performed in a reaction mixture of 4 µl cDNA, 1.25 U Taq DNA polymerase, Taq buffer with 2.5 mM Mg^{2+}, 0.5 µM upstream and downstream primers, 0.25 mM dNTPs and molecular biology grade water to a final volume of 50 µl. The PCR was performed as described in Table I. The amplified products were analyzed on 2% agarose gel, stained with ethidium bromide and quantitated by Image Quant densitometry computer program. To determine the specificities of amplified cDNAs, they were recovered from the gel, purified, subjected to sequencing reactions in presence of fluorescent-labelled nucleotides and analyzed by ABI Prism 377™ DNA sequencer (Perkin Elmer). All the obtained sequences corresponded to the expected ones (data not shown). Data obtained were confirmed by real-time PCR, using the same sets of primers described in Table I. The assays were performed in triplicate, using the ABI Prism 7000 (Applied Biosystem, CA) with SYBR Green PCR master mix (Applied Biosystem).

Western blot

Cells were resuspended in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.5% sodium deoxycholate, 150 mM sodium chloride, 1 mM EDTA, 1 mM sodium fluoride, 1 mM AEBSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate in double distilled water) and sonicated. The lysed samples were centrifuged at 10,000 rpm for 10 min, the cell extracts were aliquoted and frozen to −80°C. Normal and tumor tissue samples were homogenized in RIPA buffer by Turrax, centrifuged at 10,000 rpm for 10 min and frozen to −80°C. Protein concentrations in the cell extracts were determined by the Bradford assay.[38] Aliquot of 50 µg of cell or tissue extracts were supplemented with 5X Laemmli buffer (120 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue) containing 5% of mercaptoethanol, heated at 95°C for 5 min, electrophoresed on a 12.5% polyacrylamide gel and transferred onto nitrocellulose membranes, using the Biorad Mini Trans-Blot Cell system. The membranes were then washed with TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20) and saturated with 5% low fat milk in TBST for 2 hr at room temperature. Incubations with primary antibodies were performed for the identification of Aurora-A, -B and -C in 2.5% low fat milk in TBST at 4°C overnight. The polyclonal antibodies raised against Aurora-C (1:250) and B (1:250), and the monoclonal antibody against Aurora-A (1:200) were detected, respectively, with anti-rabbit (1:50,000) and anti-mouse (1:50,000) horseradish peroxidase conjugated secondary antibodies. Samples loadings in the different western blots were controlled with the polyclonal anti-actin (1:500). The western blots were revealed by chemiluminescence Super Signal kit from Pierce (Rockford, IL). The recombinant protein Aurora-C was produced from BL21(DE3)-pLysS after cloning the DNA encoding Aurora-C directly in the pET29 vector (Novagen). Then the recombinant protein was purified

by a Ni-NTA-agarose affinity chromatography, following the manufacturer's instructions (QuiagenSA).

Immunofluorescence

The different cell lines were grown on glass coverslips and fixed with cold methanol, rinsed twice with PBS and then treated for 3 min in 0.1% Triton X-100 in PBS at room temperature. Coverslips were then incubated with 3% BSA in PBS for 2 hr and washed thrice with PBS. The cells were incubated for 1 hr at room temperature with the different antibodies against Aurora-A (1:500), Aurora-B (1:300) and Aurora-C (1:400). After washing twice with PBS, the coverslips were incubated with a FITC-conjugated anti-mouse (1:200) or TRITC-conjugated anti-rabbit antibody (1:200) for 1 hr at room temperature, then washed with PBS and mounted in Vectashield® (Vector Laboratories, Burlingame) containing 1 μg/ml DAPI. All the coverslips were observed with a microscope Leica-DMRXA (IFR-140 GFAS-Rennes).

Cell cycle analysis

After a pulse-labelling with 30 mM BrdU at 37°C for 2 hr, the cells were collected, fixed in ice-cold ethanol and FITC-PI stained. All cell samples were analyzed for BrdU content (FITC), using an EPICS Elike Flow cytometer (Coultronics, Hialeah, FL) equipped with an argon laser (488 nm). Data analysis was carried out using Multycycle software (Phoenix Flow Systems, San Diego, CA).

Statistical analysis

All the results are expressed as the mean ± SEM of at least 3 independent experiments and values were statistically compared using the Student's *t* test or the 2-tailed Wilcoxon rank sum test, as specified in figure legends. The results were determined to be significantly different if *p* values were lower than 0.05.

Results

Aurora kinases expression in normal and transformed human thyrocytes

In this study, we analyzed the expression of all 3 members of the Aurora kinase family in normal human thyrocytes and investigated whether changes in their expression could be associated to malignant transformation. To this end, we adopted as experimental model a normal strain of human thyrocytes, the HTU5 cells, and different human cell lines derived from benign follicular adenoma (HTU42), follicular (FTC-133), papillary (B-CPAP) and anaplastic (8305C) thyroid carcinomas. The cell cycle analysis was performed with different cell lines and percentage of cells in the different phases of the cell cycle G1, S and G2/M was determined (Table II). We initially evaluated, by means of semiquantitative and real-time RT-PCR, the expression of mRNA for Aurora-A, B and C. As reported in Figure 1*a* and 1*b*, normal human thyrocytes expressed all 3 members of the Aurora family. Such expression did not change significantly in the HTU42 cells. The amount of mRNAs encoding Aurora-A and B increased, respectively, by more than 3- and 10-fold in the FTC-133, B-CPAP and 8305-C cells lines. In contrast, we observed no variation in the amount of Aurora-C mRNA in the different cell lines. (Fig. 1*b*). Western blot analysis, reported in Figure 1*c*, clearly showed in all carcinomas, but not in the follicular adenoma derived cell lines a remarkable and statistically significant ($p < 0.01$) increase in the protein level of all 3 Aurora kinases. In particular, Aurora-A and -B proteins were barely or not detectable in HTU5 and HTU42 cells, and strongly induced in FTC-133 and 8305C cell lines and less in B-CPAP cells. Aurora-C protein was detectable in normal thyroid cells and increased by (17.5 ± 1.6)-fold in FTC-133, (31.3 ± 2.1)-fold in B-CPAP and (31.5 ± 2.7)-fold in 8305C, respectively. The specificity of Aurora-C immunoreactivity was proved by performing a western blot in the presence of an Aurora-C antibody preincubated with recombinant Aurora-C protein. In panel D of Figure 1, the antibody detected a band in 8305C at the molecular weight of Aurora C. No signal was observed when the membrane was incubated with the antibody previously incubated with the recombinant protein.

TABLE II – CELL CYCLE ANALYSIS OF NORMAL HUMAN THYROCYTES (HTU5) AND CELL LINES DERIVED FROM BENIGN FOLLICULAR ADENOMA (HTU42) AND FROM PAPILLARY (B-CPAP), FOLLICULAR (FTC-133) AND ANAPLASTIC (8305C) THYROID CARCINOMAS

Cell line	% of cells ± SD		
	G1	S	G2/M
HTU5	90.2 ± 3.4	1.7 ± 0.8	8.0 ± 2.6
HTU42	74.9 ± 1.7	3.5 ± 0.3	21.6 ± 2.0
FTC-133	45.2 ± 1.5	43.8 ± 2.5	11.0 ± 1.1
B-CPAP	88.7 ± 1.9	7.0 ± 0.9	4.3 ± 1.0
8305C	43.7 ± 0.1	47.2 ± 0.1	9.1 ± 0.2

Effect of serum on normal human thyrocytes proliferation and Aurora kinases expression

To investigate whether Aurora kinases expression is cell cycle-regulated, normal human thyrocytes were exposed the HTU5 cells to high or low serum concentrations. First, we reduced the cell proliferation (S+G2/M phase) from (48.5 ± 2.7)% to (11.8 ± 2.4)% ($p < 0.01$) by cultivating them in a low serum concentration (0.3%) for 4 days (Fig. 2, panel A). The cells were then exposed to fresh medium containing 5% serum for 24 hr, which increased the number of proliferating cells to (36.3 ± 6.9)% ($p < 0.05$) (Fig. 2, panel A). On these treated cells, we thus analyzed the expression of the Aurora-A, B and C at both mRNA and protein levels. The obtained results are described in Figure 2. Four days serum deprivation induced a significant reduction in the mRNA levels for both Aurora-A and B (more than 70%). As expected, the amount of these mRNAs was restored as the serum was added to the cell culture medium. In contrast, no changes in Aurora-C mRNA were observed (Fig. 2, panels B and C). In parallel with the mRNA level, the Western blot analysis demonstrated a concomitant reduction in the protein levels for Aurora-A and B, following serum deprivation. Interestingly, also the amount of Aurora-C protein was significantly reduced (Fig. 2, panels D and E). Finally, the protein level of the 3 kinases was restored by the addition of fresh medium containing 5% FBS.

Subcellular localization of Aurora kinases in human thyroid carcinoma derived cell lines

To further characterize the expression of the Aurora kinases in human thyrocytes, we investigated their subcellular localization by means of indirect immunofluorescence microscopy. The results showed that Aurora-A localized to the centrosomes, to the poles of mitotic spindle and in spindle midbody during cytokinesis (Fig. 3, panel A). Moreover, Aurora-A staining, in some FTC-133 cells, revealed the presence of multiple centrosomes in interphasic cells and multiple polar spindles in mitotic cells (Fig. 3, panel A). Aurora-B was observed onto the centromeres during the metaphase, in the spindle midzone in anaphase and in the midbody during the cytokinesis (Fig. 3, panel A). Finally, Aurora-C colocalized with Aurora-B in the midbody of cytokinetic 8305C cells (Fig. 3, panel B). No labelling was detectable for Aurora-B and Aurora-C in interphasic phase of the cell lines.

Expression of Aurora kinases in normal and papillary carcinoma thyroid tissues

We noticed variation in the amount of Aurora kinases dependent on the tumorigenicity of the cells. To confirm the relevance of these data, we investigated the expression of the Aurora kinases in matched normal thyroid and papillary carcinomas (PTC) tissues. As reported in panel A of Figure 4, quantitative RT-PCR analysis of 7 PTC tissues demonstrated statistically significant increases

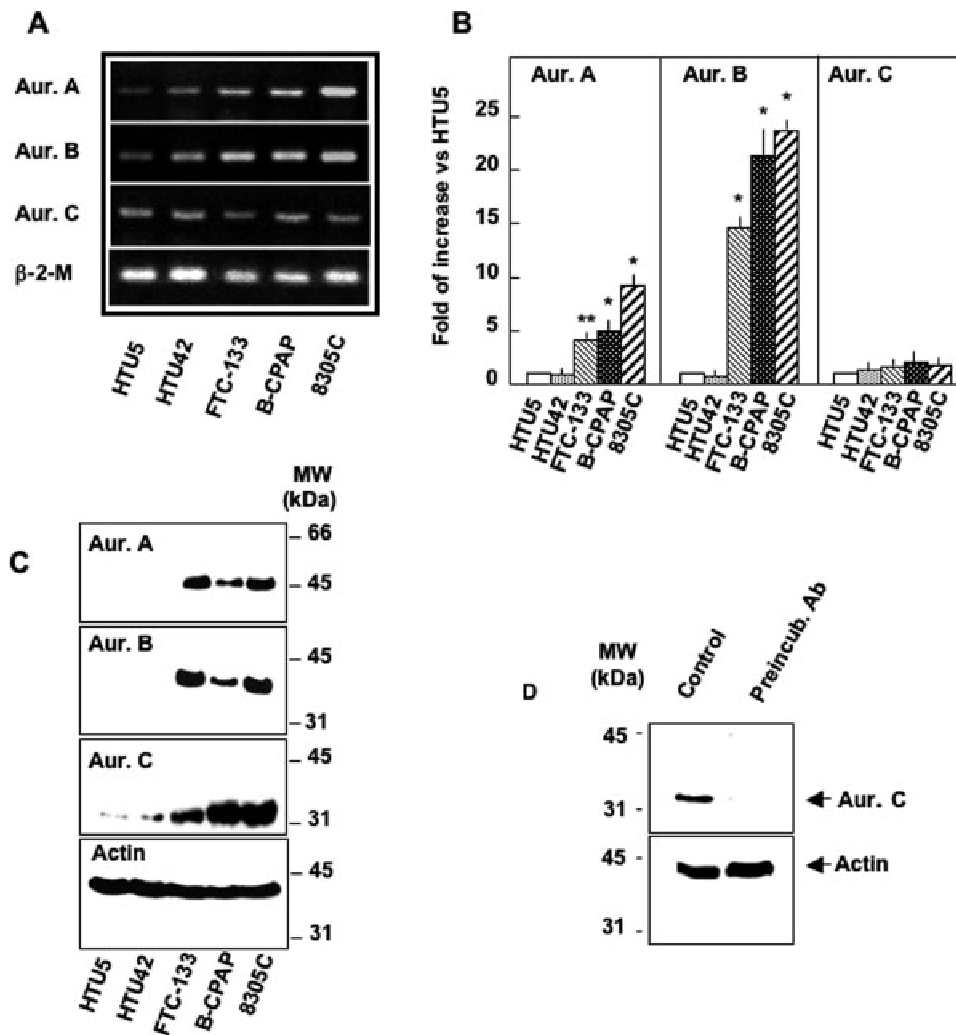


FIGURE 1 – Expression of Aurora-A, -B and -C in normal human thyrocytes and cell lines derived from benign follicular adenoma (HTU42) and from papillary (B-CPAP), follicular (FTC-133) and anaplastic (8305C) thyroid carcinomas. (*a*) Semiquantitative RT-PCR analysis of Aurora-A, -B and -C mRNAs. The different cell types were maintained in continuous monolayer cultures, expanded up to 80% of confluence, then washed and used to prepare total RNA. Aliquots of 5 μg were analyzed by RT-PCR, as described in the Material and Methods section, using specific primers described in Table I. A representative experiment out of 3 independent ones is reported. (*b*) Real-time analysis of Aurora-A, B and C mRNAs in the different cell types analyzed. Fold of mRNA variations have been calculated considering equal to 1, the Aurora kinase/β_2-microglobulin ratio observed in normal human thyrocytes. Data reported represent the mean ± SEM of 3 independent experiments. Statistical significance of data were assessed by the Student *t* test $*p < 0.01$, $**p <$ z0.05. (*c*) Western blot analysis of Aurora-A, B and C expression in the different cell lines above described. Fifty microgram of the different cell protein extracts were loaded in each lane and subjected to Western blotting as specified in the Material and Methods section, using specific antibodies against the 3 Aurora kinases and actin as protein loading control. Data shown are representative of 1 out of 3 independent experiments. (*d*) Specificity of Aurora-C immunoreactive signal. Fifty microgram of 8305C cell protein extracts were loaded in each lane and subjected to Western blotting as specified in the Material and Methods section. To evaluate the specificity of Aurora-C immunoreactivity, a Western blot was performed with the primary antibody preincubated overnight at 4°C with the recombinant Aurora-C protein or PBS alone as control.

($p < 0.01$) in the Aurora-A mRNA by (1.46 ± 0.13)-fold, with respect to normal tissues. Aurora-B mRNA was found no or slightly increased in 2 PTC and clearly induced in 5 PTC out of 7 examined. Taken all together, the data indicated a statistically significant increase of Aurora-B mRNA in PTC, with respect to normal tissues, of (1.94 ± 0.42)-fold ($p < 0.05$). On the contrary, no statistically significant modification of Aurora-C mRNA was found in the PTC, with respect to normal matched tissues (Fig. 4). Western blot experiments performed on 3 matched PTC and normal tissues showed a clear increase of all 3 Aurora proteins (Fig. 4, panel B). In particular, Aurora-A was augmented by (3.2 ± 0.4) -fold ($p < 0.01$), Aurora-B was increased by 7.1 ± 0.2 ($p < 0.01$), while Aurora-C, undetectable in normal tissues, was clearly induced in all the 3 PTC tissues.

Discussion

Despite the established role of Aurora kinases in different types of cancers,[39] information regarding their expression in normal and neoplastic thyroid tissues are very limited. In fact, only one article, published while this manuscript was in preparation, reported the overexpression of Aurora-B in thyroid undifferentiated cancer cells and tissues.[31] In the present study, we have demonstrated that normal human thyrocytes beside Aurora-B, express also the Aurora-A and C. Moreover, we showed that their expression, as already reported in other cell types, is cell cycle-dependent, being down-regulated following serum deprivation and induced by serum treatment of cell cultures (see Fig. 2).[20] In all thyroid carcinoma derived cell lines analyzed, but not in a follicular adenoma

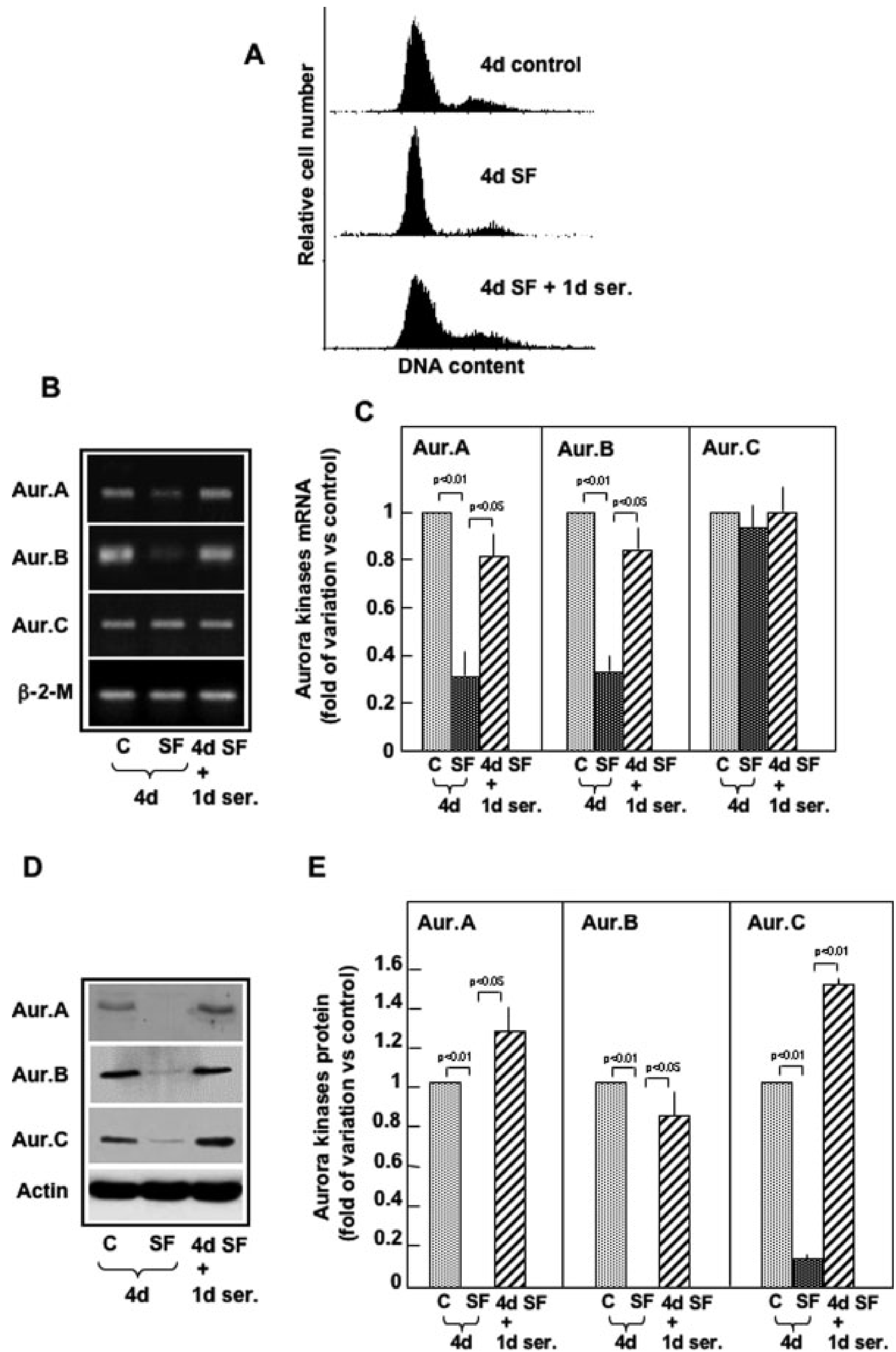


FIGURE 2 – Effect of serum deprivation on Aurora kinases expression in normal human thyrocytes. Normal human thyrocytes have been treated for 4 days in the presence of 5% (C) or 0.3% (SF) FBS. The cells, following 4 days of culture in 0.3% FBS, have been treated for additional 24 hr in media containing 5% FBS. At the end of the treatment, cells have been harvested and used to prepare total RNA or cell protein extracts to assess the Aurora kinases expression as described in the Material and Methods section. (*a*) Cell cycle profile of normal human thyrocyte in the different experimental conditions. (*b*) Semiquantitative RT-PCR analysis of Aurora kinases mRNA levels in the different culture conditions. (*c*) Quantitative analysis of Aurora kinases mRNA levels in the different culture conditions. Fold of mRNA variations have been calculated considering equal to 1 the Aurora kinase/β_2-microglobulin ratio observed in human thyrocytes culture for 4 days in control conditions (C). (*d*) Western blot analysis of Aurora kinases protein levels in the different culture conditions above described.(*e*) Densitometric analysis of Western blots for Aurora kinases in normal thyrocyte following the different treatments above described. Fold of protein variations have been calculated considering equal to 1 the Aurora kinase/actin ratio observed in human thyrocytes culture for 4 days in control conditions (C). Data reported represent the mean ± SEM of 3 independent experiments. Statistical significance of data was assessed by the Student *t* test. Data reported in panel (*a*) and (*c*) are representative of one out of 3 similar experiments.

derived one, the 3 Aurora kinases were overexpressed. In agreement, we observed the increased expression of all 3 kinases in PTC tissues, with respect to matched normal tissues. However, in the article published by Sorrentino *et al.*, Aurora-B expression was found upregulated in anaplastic but not in papillary thyroid cancer tissues.[31] This discrepancy could be explained by the fact that, as reported in our work, the range of Aurora-B mRNA increases in PTC tissues is highly variable, ranging from low level to a 4-fold increase. Thus, it is possible that analyzing a small number of PTC tissues, the upregulation of Aurora-B mRNA may not be appreciated.

All together, our data showed that the expression of Aurora-A and B is mainly regulated at the transcriptional level whereas that of Aurora-C is modulated at posttranscriptional level(s). We observed that changes in the protein levels of Aurora-A and B were mirrored by changes in the respective mRNA levels. On the contrary, conditions in which Aurora-C protein was upregulated, *i.e.,* in thyroid cancer derived cell lines or tissues, or down-regu-

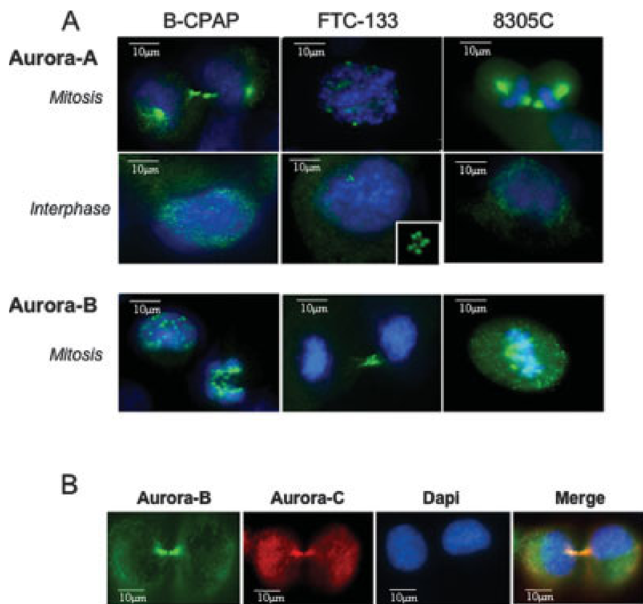
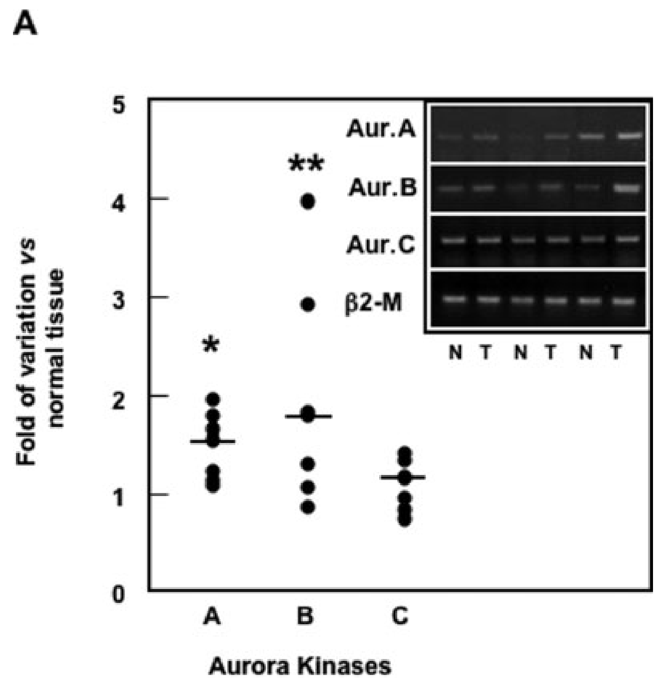


FIGURE 3 – Subcellular localization of Aurora kinases in human thyroid carcinoma cells. (*a*) The localization of Aurora-A and B in follicular (FTC-133), papillary (B-CPAP) and anaplastic (8305C) thyroid carcinoma cells was analyzed by indirect immunofluorescence microscopy in mitotic and interphasic carcinoma cells, as described in the Material and Methods section. Insert in the Aurora-A panel for intephasic FTC-133 cells shows the staining of multiple centrosomes. (*b*) Colocalization of Aurora-C and B in the midbody of cytokinetic 8305C cell. Data reported are representative of 1 out of 3 independent experiments.



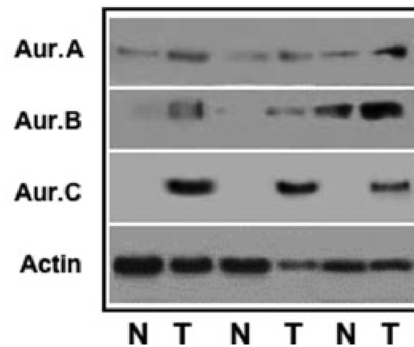


FIGURE 4 – Aurora kinases expression in matched tumor and normal tissues. (*a*) Quantitative RT-PCR analysis of Aurora-A, B and C mRNA levels in 7 papillary thyroid cancer tissues compared to normal tissues obtained from the same patients. Fold of mRNA variations have been calculated considering equal to 1 the Aurora kinase/β_2-microglobulin ratio observed in normal thyroid tissues. The insert show RT-PCR results of Aurora kinases mRNA levels in 3 out of 7 PTC analyzed. Statistical evaluation of data has been performed by the 2-tailed Wilcoxon rank sum test * $p < 0.01$, ** $p < 0.05$. (*b*) Western blot analysis of Aurora-A, B and C expression in normal and papillary carcinoma thyroid tissues. Fifty microgram of the different cell protein extracts were loaded in each lane and subjected to Western blotting as specified in the Material and Methods section, using specific antibodies against the three Aurora kinases and actin as protein loading control. Data shown are representative of 1 out of 3 independent experiments.

lated, as following serum deprivation of normal thyrocytes in culture, did not modified the mRNA amount. A possible cause of Aurora-C protein accumulation could be due to alterations in the ubiquitin–proteasome pathway controlling its degradation. For both Aurora-A and B kinases, the protein levels are regulated by periodic ubiquitination dependant proteolysis at the end of mitosis.[18,40] Although Aurora-C share a common catalytic domain with both Aurora-A and B, no studies have clearly demonstrated a cell cycle proteolytic-dependant regulation for this kinase. One could expected that the D-Box present in the catalytic domain of Aurora-C normalize the amount of protein *in vivo*. However, the molecular mechanism(s) controlling its expression in thyroid cells remains to be determined.

Recently, it has been demonstrated that Aurora-C is a new member of the chromosomal passenger proteins interacting, similarly to Aurora-B, with INCENP and survivin, thus contributing to the regulation of chromosome segregation and cytokinesis[22,41]. In this context, it is worthwhile to mention that Aurora-B and C share the highest degree of sequence overlap with about 83% sequence identity whereas the kinases C and A share about 71% sequence identity.[15] Based on the degree of structural identity, Aurora-C, therefore, is expected to be functionally closer to Aurora-B than to Aurora-A kinase. In agreement with these observations, our immunofluorescence experiments showed that in thyroid cancer cells Aurora-C colocalize with Aurora-B in the cytokinetic bridge. In the latter, we also observed the presence of Aurora-A, beside its classical localization on the centrosomes and spindle poles (see Fig. 3). These observations arise the question whether in the midbody the different kinases serve to the same function(s) or have diverse specialized functions and substrates.

Thyroid follicular neoplasms are often characterized by genetic instability; however, the information regarding the underlying biological processes are largely unknown.[7] In this context, the overexpression of Aurora-A in thyroid cancer cells reported here could be of relevance. In fact, overexpression of Aurora-A potentiates the oncogenic action of RAS, known to be implicated in human thyrocytes transformation and chromosome instability in thyroid cancer.[42] Furthermore, it has been shown that p53 may bind to the catalytic domain of Aurora-A and suppress its ability to induce centrosome amplification.[43] On the other hand, it has been documented that p53 is a substrate of Aurora-A and its phosphorylation on Ser215 leads to the inhibition of the p53 transactivating action on several genes.[44] Thus, alterations in the cross-talk between Au-

rora kinase A and p53 could be relevant in thyroid tumor progression by compromising the fidelity of chromosome segregation in thyroid cells.[8,45]

The overexpression of Aurora kinases reported here could have potential therapeutic implications. In fact, over the last few years have been identified specific inhibitors of Aurora kinases that may open a new scenario in cancer therapy, especially against those cancers that do not respond to available antimitotic agents as the anaplastic thyroid cancer.[46,47] In this context, it has been recently demonstrated that inhibition of Aurora-B expression by RNA interference or inhibition of the kinase activity significantly reduced the growth of thyroid anaplastic carcinoma cells.[23,31]

In conclusion, our study demonstrated that normal human thyrocytes express, in a cell cycle-dependent manner, all the 3 members of the Aurora kinase family and that they are overexpressed in both thyroid carcinoma cell lines and tissues. Furthermore, we showed that Aurora-A and B expression is regulated at the transcriptional level whereas posttranscriptional mechanism(s) modulate Aurora-C expression. These results suggest that the Aurora kinases may play a role in thyroid cancers and may represent a putative therapeutic target.

Acknowledgments

The microscopic observations were performed on the "Plateforme de microscopie fluorescente de l'IFR GFAS 140" in Rennes. We thank S. Dutertre for the design of the primers for Aurora-A and Aurora-B.

References

1. Sherman SI. Thyroid carcinoma. Lancet 2003;361:501–11.
2. Kinder BK. Well differentiated thyroid cancer. Curr Opin Oncol 2003;15:71–7.
3. Pasieka JL. Anaplastic thyroid cancer. Curr Opin Oncol 2003;15:78–83.
4. Sarlis NJ. Expression patterns of cellular growth-controlling genes in non-medullary thyroid cancer: basic aspects. Rev Endocr Metab Disord 2000;1:183–96.
5. Kroll TG, Sarraf P, Pecciarini L, Chen CJ, Mueller E, Spiegelman BM, Fletcher JA. *PAX8-PPAR*gamma1 fusion oncogene in human thyroid carcinoma [corrected]. Science 2000;289:1357–60.
6. Nikiforova MN, Lynch RA, Biddinger PW, Alexander EK, Dorn GW, II, Tallini G, Kroll TG, Nikiforov YE. *RAS* point mutations and PAX8-PPAR gamma rearrangement in thyroid tumors: evidence for distinct molecular pathways in thyroid follicular carcinoma. J Clin Endocrinol Metab 2003;88:2318–26.
7. Fagin JA. Minireview: branded from the start-distinct oncogenic initiating events may determine tumor fate in the thyroid. Mol Endocrinol 2002;16:903–11.
8. Shahedian B, Shi Y, Zou M, Farid NR. Thyroid carcinoma is characterized by genomic instability: evidence from *p53* mutations. Mol Genet Metab 2001;72:155–63.
9. Ouyang B, Knauf JA, Ain K, Nacev B, Fagin JA. Mechanisms of aneuploidy in thyroid cancer cell lines and tissues: evidence for mitotic checkpoint dysfunction without mutations in BUB1 and BUBR1. Clin Endocrinol (Oxf) 2002;56:341–50.
10. Corso C, Ulucan H, Parry EM, Parry JM. Comparative analysis of two thyroid tumor cell lines by fluorescence in situ hybridization and comparative genomic hybridization. Cancer Genet Cytogenet 2002;137: 108–18.
11. Dettori T, Frau DV, Garcia JL, Pierantoni G, Lee C, Hernandez JM, Fusco A, Morton CC, Vanni R. Comprehensive conventional and molecular cytogenetic characterization of B-CPAP, a human papillary thyroid carcinoma-derived cell line. Cancer Genet Cytogenet 2004; 151:171–7.
12. Hahn WC. Cancer: surviving on the edge. Cancer Cell 2004;6:215–22.
13. Lingle WL, Barrett SL, Negron VC, D'Assoro AB, Boeneman K, Liu W, Whitehead CM, Reynolds C, Salisbury JL. Centrosome amplification drives chromosomal instability in breast tumor development. Proc Natl Acad Sci USA 2002;99:1978–83.
14. Nigg EA. Mitotic kinases as regulators of cell division and its checkpoints. Nat Rev Mol Cell Biol 2001;2:21–32.
15. Bischoff JR, Plowman GD. The Aurora/Ipl1p kinase family: regulators of chromosome segregation and cytokinesis. Trends Cell Biol 1999;9:454–9.
16. Kimura M, Kotani S, Hattori T, Sumi N, Yoshioka T, Todokoro K, Okano Y. Cell cycle-dependent expression and spindle pole localization of a novel human protein kinase, Aik, related to Aurora of Drosophila and yeast Ipl1. J Biol Chem 1997;272:13766–71.
17. Giet R, Prigent C. The *Xenopus laevis* Aurora/Ip11p-related kinase pEg2 participates in the stability of the bipolar mitotic spindle. Exp Cell Res 2000;258:145–51.
18. Arlot-Bonnemains Y, Klotzbucher A, Giet R, Uzbekov R, Bihan R, Prigent C. Identification of a functional destruction box in the *Xenopus laevis* Aurora-A kinase pEg2. FEBS Lett 2001;508:149–52.
19. Adams RR, Eckley DM, Vagnarelli P, Wheatley SP, Gerloff DL, Mackay AM, Svingen PA, Kaufmann SH, Earnshaw WC. Human INCENP colocalizes with the Aurora-B/AIRK2 kinase on chromosomes and is overexpressed in tumour cells. Chromosoma 2001;110: 65–74.
20. Carmena M, Earnshaw WC. The cellular geography of Aurora kinases. Nat Rev Mol Cell Biol 2003;4:842–54.
21. Vagnarelli P, Earnshaw WC. Chromosomal passengers: the four-dimensional regulation of mitotic events. Chromosoma 2004;113: 211–22.
22. Yan X, Cao L, Li Q, Wu Y, Zhang H, Saiyin H, Liu X, Zhang X, Shi Q, Yu L. Aurora-C is directly associated with Survivin and required for cytokinesis. Genes Cells 2005;10:617–26.
23. Zhou H, Kuang J, Zhong L, Kuo WL, Gray JW, Sahin A, Brinkley BR, Sen S. Tumour amplified kinase STK15/BTAK induces centrosome amplification, aneuploidy and transformation. Nat Genet 1998; 20:189–93.
24. Tanner MM, Grenman S, Koul A, Johannsson O, Meltzer P, Pejovic T, Borg A, Isola JJ. Frequent amplification of chromosomal region 20q12-q13 in ovarian cancer. Clin Cancer Res 2000;6:1833–9.
25. Takahashi T, Futamura M, Yoshimi N, Sano J, Katada M, Takagi Y, Kimura M, Yoshioka T, Okano Y, Saji S. Centrosomal kinases, HsAIRK1 and HsAIRK3, are overexpressed in primary colorectal cancers. Jpn J Cancer Res 2000;91:1007–14.
26. Sakakura C, Hagiwara A, Yasuoka R, Fujita Y, Nakanishi M, Masuda K, Shimomura K, Nakamura Y, Inazawa J, Abe T, Yamagishi H. Tumour-amplified kinase BTAK is amplified and overexpressed in gastric cancers with possible involvement in aneuploid formation. Br J Cancer 2001;84:824–31.
27. Miyoshi Y, Iwao K, Egawa C, Noguchi S. Association of centrosomal kinase STK15/BTAK mRNA expression with chromosomal instability in human breast cancers. Int J Cancer 2001;92:370–3.
28. Kamada K, Yamada Y, Hirao T, Fujimoto H, Takahama Y, Ueno M, Takayama T, Naito A, Hirao S, Nakajima Y. Amplification/overexpression of Aurora-A in human gastric carcinoma: potential role in differentiated type gastric carcinogenesis. Oncol Rep 2004; 12:593–9.
29. Katayama H, Ota T, Jisaki F, Ueda Y, Tanaka T, Odashima S, Suzuki F, Terada Y, Tatsuka M. Mitotic kinase expression and colorectal cancer progression. J Natl Cancer Inst 1999;91:1160–2.
30. Ota T, Suto S, Katayama H, Han ZB, Suzuki F, Maeda M, Tanino M, Terada Y, Tatsuka M. Increased mitotic phosphorylation of histone H3 attributable to AIM-1/Aurora-B overexpression contributes to chromosome number instability. Cancer Res 2002;62:5168–77.
31. Sorrentino R, Libertini S, Pallante PL, Troncone G, Palombini L, Bavetsias V, Spalletti-Cernia D, Laccetti P, Linardopoulos S, Chieffi P, Fusco A, Portella G. Aurora-B overexpression associates with the thyroid carcinoma undifferentiated phenotype and is required for thyroid carcinoma cell proliferation. J Clin Endocrinol Metab 2005;90: 928–35.
32. Smanik PA, Fithian LJ, Jhiang SM. Thyroid peroxidase expression and DNA polymorphisms in thyroid cancer. Biochem Biophys Res Commun 1994;198:948–54.
33. Fabien N, Fusco A, Santoro M, Barbier Y, Dubois PM, Paulin C. Description of a human papillary thyroid carcinoma cell line. Morphologic study and expression of tumoral markers. Cancer 1994;73: 2206–12.
34. Smit JW, van der Pluijm G, Romijn HA, Lowik CW, Morreau H, Goslings BM. Degradation of extracellular matrix by metastatic follicular thyroid carcinoma cell lines: role of the plasmin activation system. Thyroid 1999;9:913–19.
35. Curcio F, Ambesi-Impiombato FS, Perrella G, Coon HG. Long-term culture and functional characterization of follicular cells from adult normal human thyroids. Proc Natl Acad Sci USA 1994;91: 9004–8.
36. Perrella G, Fabbro D, Damante G, Di Loreto C, Beltrami CA, Curcio F, Ambesi-Impiombato FS. Expression of differentiation markers in cultured cells from various thyroid diseases. Thyroid 1997;7:817–21.
37. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156–9.

38. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
39. Katayama H, Brinkley WR, Sen S. The Aurora kinases: role in cell transformation and tumorigenesis. Cancer Metastasis Rev 2003;22: 451–64.
40. Littlepage LE, Ruderman JV. Identification of a new APC/C recognition domain, the A box, which is required for the Cdh1-dependent destruction of the kinase Aurora-A during mitotic exit. Genes Dev 2002;16:2274–85.
41. Sasai K, Katayama H, Stenoien DL, Fujii S, Honda R, Kimura M, Okano Y, Tatsuka M, Suzuki F, Nigg EA, Earnshaw WC, Brinkley WR, et al. Aurora-C kinase is a novel chromosomal passenger protein that can complement Aurora-B kinase function in mitotic cells. Cell Motil Cytoskeleton 2004;59:249–63.
42. Tatsuka M, Sato S, Kitajima S, Suto S, Kawai H, Miyauchi M, Ogawa I, Maeda M, Ota T, Takata T. Overexpression of Aurora-A potentiates HRAS-mediated oncogenic transformation and is implicated in oral carcinogenesis. Oncogene 2005;24: 1122–7.
43. Chen SS, Chang PC, Cheng YW, Tang FM, Lin YS. Suppression of the STK15 oncogenic activity requires a transactivation-independent p53 function. EMBO J 2002;21:4491–9.
44. Liu Q, Kaneko S, Yang L, Feldman RI, Nicosia SV, Chen J, Cheng JQ. Aurora-A abrogation of p53 DNA binding and transactivation activity by phosphorylation of serine 215. J Biol Chem 2004;279: 52175–82.
45. Parry EM, Ulucan H, Wyllie FS, Wynford-Thomas D, Parry JM. Segregational fidelity of chromosomes in human thyroid tumour cells. Chromosoma 1998;107:491–7.
46. Harrington EA, Bebbington D, Moore J, Rasmussen RK, Ajose-Adeogun AO, Nakayama T, Graham JA, Demur C, Hercend T, Diu-Hercend A, Su M, Golec JM, et al. VX-680, a potent and selective small-molecule inhibitor of the Aurora kinases, suppresses tumor growth in vivo. Nat Med 2004;10:262–7.
47. Hata T, Furukawa T, Sunamura M, Egawa S, Motoi F, Ohmura N, Marumoto T, Saya H, Horii A. RNA interference targeting Aurora kinase A suppresses tumor growth and enhances the taxane chemosensitivity in human pancreatic cancer cells. Cancer Res 2005;65:2899–905.