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Assessment of Aurora A kinase expression in breast cancer: A tool for early diagnosis?

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Abstract. Aurora A kinase is overexpressed in many cancers but the status of this protein in the breast cancer often varies. We investigate the expression and localization of Aurora A protein in relation with tumor emergence and progression in breast cancer. Aurora A kinase status was evaluated in 107 patients using immunohistochemistry. The experimental findings showed that high expression of the Aurora A protein was correlated with elevated nuclear grade, low expression of progesterone receptor and positive nodal status. The experimental results showed also that the localization of this kinase shifts from cytoplasm in non malignant adjacent tissue to both cytoplasmic and nuclear compartments in tumoral tissue, suggesting an oncogenic role of the nuclear accumulation. We have, furthermore, detected the overexpression of this protein in non malignant adjacent tissue. The expression of the Aurora A kinase in non malignant tissue may represent an earlier diagnosis tool for breast cancer.

Keywords: Aurora A, breast cancer, immunohistochemistry

1. Introduction

The Aurora A protein is a Serine/Threonine kinase, with 403 amino acids and 46 kilodaltons (Kda) as molecular weight [1]. This kinase plays a crucial role during mitosis [2,3]. It acts as a key regulator of multiple mitotic events: centrosome maturation and separation [4], regulation of spindle assembly [5,6], equal segregation of chromosomes and cytokinesis [7]. Aurora A is a cell-cycle regulated protein [8], and this centrosome-associated kinase is active and expressed at a high level during G2-M phases [9–11]. This protein is degraded at the end of mitosis [12–15]. In normal tissue Aurora A is restrained to the centrosomes while in late phases of mitosis it is located at the spindle poles. Overexpression of this kinase alters centrosome number and function leading to aberrant mitotic spindle, missegregation of chromosomes and cellular transformation [16]. Errors in the mitotic process are considered to be one of the major causes of genetic instability that hallmarks cancer [17].

Hence Aurora A is an oncogenic protein encoded by the oncogene STK 15 located on chromosome 20q13.2, which is a highly amplified region in various human tumors such as the esophageal squamous cell carcinoma [18], the colorectal carcinomas [19], the ovarian and gastric cancers [21], human gliomas [22] and breast cancer [23,24]. Moreover, mRNA of STK15 is reported to be enhanced in various cancers [23–26]. Polymorphisms in this gene are associated with increased risk of several cancers [27–31] particularly in breast cancer [32].

Aurora A protein is frequently overexpressed in several human cancers: lung [33], head and neck [34], ovarian [35,36], colon [37], prostate [38], pancr-

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eas [39], esophagus [40] and breast one [41], as shown by immunohistochemistry (IHC).

In breast cancer, aneuploidy (abnormal number of chromosomes) is highly prevalent, probably associated with Aurora A; which has been initially identified as BTAK: Breast Tumor Amplified Kinase [1,42]. Breast cancer is the most common type of cancer for women, it's a hormone related tumor with various grade and stage presentations. Some studies were conducted to assess rate expression of Aurora A protein and to reveal its role in breast cancer [41,43–45]. The aim of this study is to investigate the expression level and subcellular localization of Aurora A and to establish if there is any relationship with clinical variables in breast cancer.

2. Materials and methods

2.1. Clinical tissue sample

107 archival paraffin-embedded tumor sections were collected at the Oncology Institute of Saleh Azaïez (ISA). They have been obtained from patients who underwent surgery with pathologically confirmed breast cancer, and admitted to the institute between 1999 and 2010. The following clinical data were collected: age, histological subtype, tumor grade (SBR: Scarff Bloom and Richardson) tumor size, progesterone and estrogen receptors expression (RP, RE), nodal status, nuclear grade and finally, mitotic index. The patients' characteristics are shown in Table 1. The study protocol was approved by the head of the department of histopathology at the ISA institute.

Table 1
Patient characteristics ($n = 107$)

Characteristic	Number of patients [%]
Age [years]	
Median	45
Range	24–85
Histological subtype	
Invasive ductal carcinoma	95 [88.8]
Lobular carcinoma	8 [7.5]
Other histology	4 [3.7]
SBR	
I	15 [14]
II	50 [46.7]
III	42 [39.3]
Tumor Size	
T1 [$<$ 20 MM]	14 [14.2]
T2 [$\geqslant$ 20MM and $\leqslant$ 50MM]	72 [72.7]
T3 [$>$ 50MM]	13 [13.1]
Unknown	8
Receptor status	
RE+ RP+	38 [56.7]
RE- RP-	17 [25.4]
RE+ RP-	7 [10.4]
RE- RP+	5 [7.5]
Unknown	40
Nodal status	
N+	56 [70.9]
N-	23 [29.1]
Unknown	28
Nuclear grade	
I	16 [15.7]
II	55 [53.9]
III	31 [30.4]
Unknown	5
Mitotic index [nb of mitotic cell/ 10 HPFs^{+}]	
1 [0 – 5]	40[41.0]
2 [6 – 10]	24[35.0]
3 [$>$ 11]	33[34.0]
Unknown	10

$^{+}$HPFs: high-power fields.

2.2. Antibodies

For this study we used the 6F11 monoclonal antibody for Estrogen Receptor (Novocastra Laboratories, UK) and the PgR636 monoclonal antibody for Progesterone Receptor (Novocastra Laboratories, UK). For Aurora A protein detection we used a mouse monoclonal antibody (clone 35c1), which was developed against the non catalytic N-terminal domain of the kinase; this clone was tested on several breast cancer cell lines: MCF-7, S68, T47D, MDA-MB-468 and SK-Br-3, at the UMR6061 in the Institute of Genetics and Development of Rennes, France [46].

2.3. Western blot

The specificity of the Aurora A antibody has been tested on different human cell lines lysates: Hek 293, Hela, RCC4^{+} and RCC4^{-} (ATCC, USA), and also on the recombinant human His-Aurora A protein experienced with an increasing concentration. Lysate from each cell line was separated by 12% SDSPAGE, and the migration was made, in parallel, with the molecular weight standards (low range, Bio-Rad Laboratories, CA); then we transferred separated proteins to nitrocellulose membrane (Hybond Amersham, GE Healthcare, UK). The membranes were blocked in 5% skim milk in a TBS-T. We probed with the Aurora A antibody in 2.5% skim milk in a TBS-T. Finally, we incubated with a secondary antibody: peroxidase-conjugated secondary mouse antibody (Jackson ImmunoResearch Laboratories,UK). The protein band was visualized by enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech, FR) West Pico or Dura (Pierce, Rockford, IL).

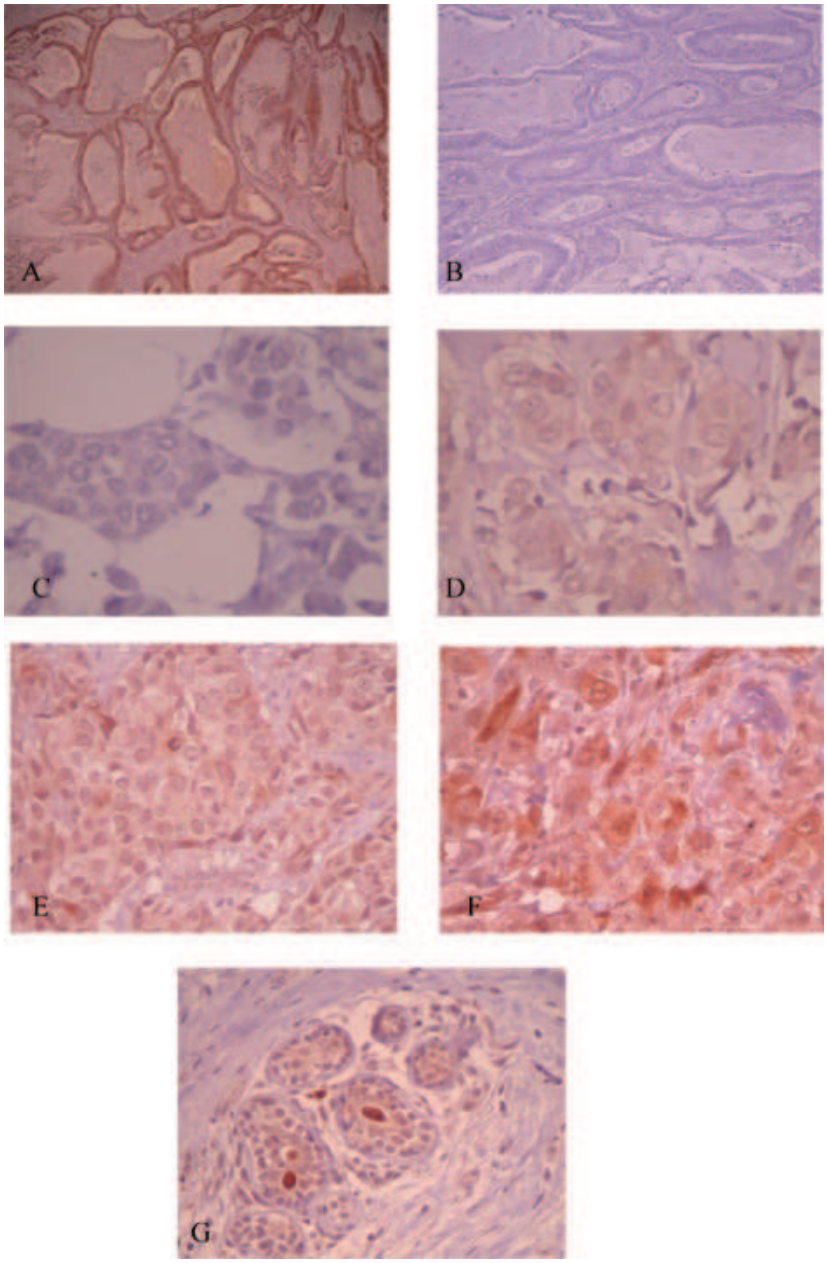

Fig. 1. A and B: Immunostaining of colorectal carcinoma (x400): (A): with anti-Aurora-A antibody: positive control, (B): without anti-Aurora-A antibody: négative control. C, D, E and F: Aurora-A immunostaining in human mammary malignant tissue (x400), brown color indicates antibody binding and intensity of staining is: (C): 0 negative staining, (D): +1 weakly positive staining, (E): +2 moderately positive staining, (F): +3 strongly positive staining. G: Aurora-A immunostaining in lobular mammary non malignant tissue (x400). By using the autologous antibody of Aurora-A protein on a histological section of non malignant lobular mammary, adjacent to the tumor tissue, we observe a staining of this protein in the cytoplasm. (Colours are visible in the online version of the article; http://dx.doi.org/10.3233/DMA-120947)

2.4. *Immunohistochemical staining*

3 μm sections were prepared for each case and afterwards stained with Hematoxylin Eosin (H&E), intended for histological examination. When the patient's condition requires further investigation, estrogen and progesterone receptors status (ER and PR) were determined.

Briefly, we deparaffinized sections in xylene and we rehydrated them through graded alcohols, then we used

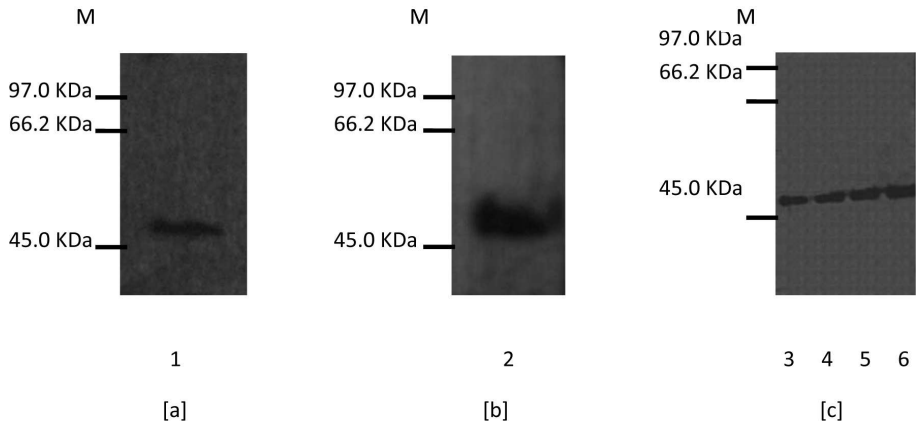

Fig. 2. Western blots with the 35C1 antibody on cell line and purified recombinant human His-Aurora-A protein. (a) lane 1: Hek 293 cells; (b) lane 2: Hela cells; (c) with increasing concentration of the recombinant protein His-Aurora-A: lane 3: 0,25 μg, lane 4: 0,5 μg, lane 5: 0,75 μg, lane 6: 1 μg, lane M: Protein molecular weight markers.

Table 2
Details about slides ($n = 107$)

Type of the tissue	Number of slides
DCIV + IS + Non malignant tissue	54
DCIV + Non malignant tissue	33
DCIV + IS	5
DCIV	11
IS + Non malignant tissue	4

a Novolink polymer detection system (RE7150-K, Leica, TN) as recommended in the instruction manual. The slides were incubated with the primary antibody Aurora A at 1:50 overnight in a humidity tray; and a slid of colorectal carcinoma was included in each batch as a positive control for Aurora A (Fig. 1A). A negative control was performed by omitting the primary antibody and did not produce any visible background staining (Fig. 1B).

The evaluation used for intensity of staining and subcellular localization was performed in a blinded manner. Intensity was scored from 0: no staining, +1: weak, +2: moderate to +3: strong (Fig. 1C, D, E, F). We also considered: range of intensity with the percentage of cell stained when the tumor was heterogeneous. When less than 10% of cell showed staining, it was scored as negative. Altogether, these data allowed to establish a semi-qualitative score calculated as following: % of cell (score1) + % of cell (score2) + % of cell (score3) = final score.

2.5. Statistical analysis

The analysis of the results involving the expression of the Aurora A protein and the various histological and clinical parameters were made by using the chi-square and regression linear performed with the program Statistical Package for Social Sciences (SPSS 13.0) and Epi info 6. We considered that results were statistically significant when p values were < 0.05, asterisks indicate $p < 0.05$ (*) and $p < 0.01$ (**).

3. Results

Since antibody specificity is of great importance in immunohistochemistry, we analyzed the specificity of the Aurora A antibody prepared by the laboratory. The specificity of the Aurora A antibody was tested by western blot performed with recombinant Aurora A protein and cellular extracts. A single specific band was detected at 46 Kda and 50 Kda corresponding respectively to cellular and to recombinant protein (Fig. 2).

The invasive Ductal Carcinoma (DCIV) and the 'In Situ' (IS) tumors cells were analyzed along with the adjacent normal mammary tissue in 54 slides, but in most of the cases only two types of tissues were available. Details concerning slides are in Table 2.

Using the autologous antibody, Aurora A protein expression was tested by IHC on breast tissue. Overexpression was observed in 92.2% for Invasive Ductal Carcinoma (DCIV) and in 91.9% for '*In Situ*' Ductal Carcinoma (IS). In the case of DCIV, Aurora A overexpression was observed in 83.2% of the cases in both nucleus and cytoplasm, while 4.2% in only nucleus and 12.6% in only cytoplasm. Whereas in IS the percentages are respectively: 79.3%, 3.5% and 17.2%. The results present no significant differences between Aurora A expression and localization in DCIV and IS.

Table 3
Comparison of Aurora A expression and localization in malignant and non malignant tissues

Aurora A	Malignant	Non malignant
Overexpression	99 [92.5%]	77 [84.6%] £
Negative expression	8 [7.5%]	14 [15.4%] £
Nuclear & Cytoplasmic	82 [82.8 %]	37 [48%] υ
Nuclear Or Cytoplasmic	17 [17.2%]	40 [52 %] υ
Only Nuclear	4	8
Only cytoplasmic	13	32

£: $p = 0.07$. υ: $p = 0.000001^{**}$, OR $= 5.21$, RR $= 2.31$.

Table 4
Association between Aurora A expression in malignant tissue and pathologic parameters

Pathologic parameters	Aurora A expression p
Age	0.30
SBR	0.06
Tumor size	0.20
RE	0.26
RP	0.01*
Nodal status	0.02*
Nuclear grade	0.02*
Mitotic index	0.37

Altogether, the tumoral tissue has protein overexpression in 92.5% (Table 3). When it was possible, the normal adjacent mammary tissues were included in the study and we observed a weak and a moderate but not a strong Aurora A expression in non malignant breast tissue with a rate of 84.6% (Fig. 1G). No significant differences between Aurora A expression in malignant and non malignant tissue is observed $p = 0.07$, even if this protein seems less frequently expressed in non malignant tissue (Table 3).

The subcellular localization of the protein in malignant tissue was both cytoplasmic and nuclear (Fig. 1F) in 82.8 % of the cases. In 4.1% of the cases the expression observed was only nuclear and in 13.1% was only cytoplasmic (Fig. 1D). In normal tissues the distribution of the protein was as follows: 48% nuclear and cytoplasmic, 41.6% cytoplasmic only and 10.4% nuclear only.

The Aurora A localization profile in both nucleus and cytoplasm is correlated with malignant tissue rather than with non malignant tissue $p = 0.000001^{**}$, OR $=$ 5.21, RR $= 2.31$; (Table 3).

The statistical study showed a correlation between a high Aurora A expression with an elevated nuclear grade ($p = 0.021^{*}$), as well as a low progesterone receptor positivity ($p = 0.016^{*}$). Finally we found an association between high Aurora A expression in malignant tissue and positive nodal status ($p = 0.021^{*}$) (Table 4).

4. Discussion

In this study, evidence has been provided for an overexpression of Aurora A in tumoral breast cancer tissue: DCIV and IS, in agreement with the previous studies [41,43–45]. The overexpression of this protein may be due to gene amplification which was reported in several studies [23,24], but the increase in the rate of the protein may also be explained by the aberration of the degradation systems of this&^{2} kinase [12–15].

Only the work of Nadler et al. [41] mentioned the presence of the protein in the nucleus in breast cancer, the other authors found only a cytoplasmic localization. We found that this protein is often expressed in nucleus and cytoplasm at the same time. In our study, we obtained a relationship between a high expression of Aurora A kinase and an elevated nuclear grade on in agreement with the works of Royce et al. [45] and Nadler et al. [41], on the one hand, and with nodal status positivity as well as with low progesterone receptor expression in conformity with the works of Nadler et al., on the another hand. The relationship between Aurora A expression and nuclear grade is expected according to Aurora A function.

The overexpression of the Aurora A kinase by the non-malignant tissue was reported by Hoque et al. [44], but not by Tanaka et al. [43]. In this study, we find that non tumoral tissue overexpresses this protein at a frequency of 84.6%. Thus observation goes to the logic of the malignant processing; the tissue being morphologically healthy could have already begun its cancerization process. Indeed, we observed correlation between tumoral and normal tissues from the same patient for the Aurora A expression. However, there are differences in protein localization observed between tumor and normal tissues, since the dual expression in nucleus and cytoplasm is associated with tumor. Less than 50% of normal tissue display such profile expression $p = 0.000001^{**}$, OR $= 5.21$, RR $= 2.31$. It is possible that the presence of the protein in both nuclear and cytoplasmic compartments corresponds to an early or established tumoral status. The shift of the protein from the cytoplasmic expression to simultaneously expression in both nucleus and cytoplasm can be explained by several mechanisms: taking into $\neq$ consideration that the STK15 gene amplification is reported in breast cancer in several studies [23,24], we can suggest that the alternative splicing is altered and could change the balance of the protein into nuclear isoforms during the transformation [31,47]. However it remains unclear how the splicing occurs in the context of gene amplifi-

cation. From another point of view, during cancerization process, the cell begins its transformation by expressing cytoplasmic isoforms before acquiring a more aggressive aspect triggering the synthesis of the nuclear isoforms. We can also assume that the protein is expressed with all its isoforms as soon as the process of malignancy is initiated but remains healthy in appearance; however, when the protein is exported to the nucleus, the cell acquires a malignant phenotype. We can eventually propose that early in the malignant process, cell keeps an aberrant degradation system, which recognizes and destroys the nuclear isoforms; as the cancer process advances progressively; the degradation machinery of the protein no longer recognizes all of its isoforms [12–15] that is why the nuclear isoforms appear. Otherwise, we can also suppose that in cancer tissue, aurora A is differentially modified or regulated, leading to its mislocalization in the nucleus. We think that this result asks for more investigation, in order to better understand the shift in the subcellular localization during malignant transformation.

To recapitulate, we propose the following scenario: a first deregulation step in apparent healthy mammary tissue leads to a high level translation of Aurora A in the cytoplasm, in a second step, it is exported to nucleus; this process could be followed by a shift to tumor status which would be characterized by the presence of Aurora A protein in both nucleus and cytoplasm.

In fact, the overexpression and the localization of this protein should be evaluated in order to be used as a tool of early screening even before the cell and tissue change their morphology and earlier than the first stage of cancer is evident in histology. The screening of this pathology uses mammography with Magnetic Resonance Imaging (MRI) [48] or Ultrasound (US) when the patient has a dense breast tissue [49,50]. In addition, we need to analyze a "real" normal mammary tissue from healthy women and make a long-term survey to inspect the upcoming of every found profile. Since Aurora A is present in a normal cell only during the G2 and Mitosis phases, and does not exist in quiescent cells [51,52], it would be interesting to understand its mechanisms of deregulation during carcinogenesis.

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