Aurora A is a prognostic marker for breast cancer arising in BRCA2 mutation carriers

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Abstract

Overexpression of the Aurora A kinase has been shown to have prognostic value in breast cancer. Previously, we showed a significant association between AURKA gene amplification and BRCA2 mutation in breast cancer. The aim of this study was to assess the prognostic impact of Aurora A overexpression on breast cancer arising in BRCA2 mutation carriers. Aurora A expression was evaluated by immunohistochemistry on breast tumour tissue microarrays from 107 BRCA2 999del5 mutation carriers and 284 of sporadic origin. Prognostic value of Aurora A nuclear staining was estimated in relation to clinical markers and adjuvant treatment, using multivariate Cox’s proportional hazards ratio regression model. BRCA2 wild-type allele loss was measured by TaqMan in BRCA2 mutated tumour samples. All statistical tests were two sided. Multivariate analysis of breast cancer-specific survival, including proliferative markers and treatment, indicated independent prognostic value of Aurora A nuclear staining for BRCA2 mutation carriers (hazards ratio 5 7.06; 95% confidence interval 5 1.23–40.6; p 5 0.028). Poor breast cancer-specific survival of BRCA2 mutation carriers was found to be significantly associated with combined Aurora A nuclear expression and BRCA2 wild type allele loss in tumours (p < 0.001). Multivariate analysis indicated independent prognostic value of both positive Aurora A nuclear staining for BRCA2 mutation carriers (hazards ratio = 7.06; 95% confidence interval = 1.23–40.6; p = 0.028) and BRCA2 wild type allele loss in tumours (p < 0.001). Multivariate analysis indicated independent prognostic value of both positive Aurora A nuclear staining (hazards ratio = 10.09; 95% confidence interval = 1.19–85.4, p = 0.034) and BRCA2 wild type allele loss (hazards ratio = 9.63; 95% confidence interval = 1.81–51.0, p = 0.008) for BRCA2 mutation carriers. Aurora A nuclear expression was found to be a significant prognostic marker for BRCA2 mutation carriers, independent of clinical parameters and adjuvant treatment. Our conclusion is that treatment benefits for BRCA2 mutation carriers and sporadic breast cancer patients with Aurora A positive tumours may be enhanced by giving attention to Aurora A targeted treatment.

Keywords: breast cancer; Aurora A; BRCA2; prognosis; wild type allele loss; adjuvant treatment

Introduction

The Aurora protein kinase A, encoded by the AURKA gene, is a member of a serine/threonine family that is important in regulation of the cell cycle [1,2]. Aurora A has a role in centrosome maturation and separation, mitosis entry, formation and function of the bipolar spindle, alignment of chromosomes in metaphase and cytokinesis [3–6]. The BRCA2 protein, on the other hand, is a key player in regulating homologous recombination by direct interaction with the RAD51 recombinase, in addition to being involved in protection of stalled DNA replication forks [7,8]. Absence of BRCA2 has been shown to lead to centrosome amplification as well as hampering cell division [9,10]. Aurora A accumulation and hereditary BRCA2 mutations have separately been associated with aneuploidy, centrosomal amplification, G2/M transition induction and failures or delay in completing cytokinesis in breast tumour cells [9–14]. These
observations indicate that BRCA2 and Aurora A abnormalities may interact at the early stages of breast tumourigenesis. Aurora A overexpression has been shown to be an early mammary tumourigenesis factor [15,16] and could, therefore, have a major role in BRCA2-associated tumourigenesis. In our previous study, we found that AURKA gene amplification associated significantly with familial breast tumours among carriers of the BRCA2 999del5 mutation, a founder mutation in the Icelandic population with a prevalence of 0.6% [17,18]. Our results suggested an increased risk of Aurora A-associated tumourigenesis in BRCA2 mutation carriers, probably through abnormalities in DNA damage response and control of cell division.

Several studies based on Aurora A mRNA and immunohistochemical (IHC) expression analysis on large cohorts of breast cancer patients have shown indications of Aurora A overexpression as a strong independent prognostic marker for breast cancer [19–26]. None of these studies, however, focused on familial breast cancer. Aurora A overexpression has recently been shown to downregulate BRCA2 expression in breast, pancreatic and ovarian cell lines [27]. Furthermore, Aurora A modulates BRCA2-directed homologous recombination by inhibition of RAD51 recruitment to DNA double-strand breaks [28]. Therefore, it is of interest to analyse the impact of Aurora A overexpression in breast cancers with a hereditary BRCA2 mutation. About half of familial BRCA2 999del5 breast carcinomas have lost their wild-type allele [29] and the same applies to pancreatic tumours [30]. How BRCA2 wild-type allele loss may influence breast cancer prognosis in combination with Aurora A overexpression has until now been unknown.

The aim of the current study was to define a possible prognostic impact of Aurora A expression on breast cancer arising in BRCA2 mutation carriers using immunohistochemistry on tissue microarrays (TMAs). We found Aurora A to be a significant prognostic marker for breast cancer and independent of treatment and clinical parameters among BRCA2 mutation carriers. Poor breast cancer-specific survival was found to be associated with combined Aurora A nuclear staining and BRCA2 wild type allele loss in BRCA2 breast tumours.

Patients and methods

Patients

The study cohort included 391 well-defined breast cancer patients. The patients were previously screened for the local germ-line BRCA2 999del5 and BRCA1 5193G→A founder mutations and tumours analysed for IHC markers such as Ki-67, progesterone receptor (PR) and oestrogen receptor (ER) as previously described [29,31]. Patients negative for the local BRCA1 and BRCA2 germline mutations were defined as sporadic cases. Of the 391 primary breast tumours, 107 had the hereditary BRCA2 999del5 mutation and 284 were of sporadic origin. All cases in the study were negative for the local BRCA1 mutation. The patients included in this study were diagnosed with breast cancer between the years 1952 and 2004 (median year 1992) and mean follow-up time was 15.8 years. Data on clinical parameters (including tumour size, nodal status, tumour grade and DNA index) were obtained from the Department of Pathology, National University Hospital, Reykjavik, Iceland. Tumours were classified as diploid if the DNA index was 1.00 ± 0.15 and aneuploid if the DNA index was <0.85 or >1.15 [32,33]. Adjunct treatment information was collected for chemotherapy, radiotherapy and endocrine therapy for the BRCA2 breast cancer subgroup from the Department of Oncology, National University Hospital. Chemotherapy was given according to the standard at the time. Of the 57 patients treated with chemotherapy, 60% received anthracyclines, 40% received cyclophosphamide, methotrexate and 5-fluorouracil (CMF) and 9% received taxane-based chemotherapy. Endocrine therapy consisted of tamoxifen and/or aromatase inhibitor. Radiotherapy consisted of 50 Gy through 5 weeks to the breast after sector resection and 46 Gy through 4.5 weeks to the chest wall and loco-regional lymph nodes after mastectomy. Information on patient age, date of diagnosis and survival were obtained from the population-based Icelandic Cancer Registry [34]. Sporadic cases were matched to the BRCA2 mutation carriers based on age and year of breast cancer diagnosis ±2 years. This work was carried out according to permits from the Icelandic Data Protection Commission (2006050307) and Bioethics Committee (VSNb2006050001/03-16).

Aurora A protein immunohistochemistry

TMAs were constructed by selecting viable and representative regions enriched for tumour cells from formalin-fixed and paraffin-embedded tumour tissues as previously described [29,31]. A total of 14 TMA slides representing three core samples from each case were stained by IHC with the Aurora A [35C1] antibody (GeneTex, cat. GTX13824) at a dilution of 1:50. Heat-induced antigen retrieval was achieved in
a 10 mM citrate buffer pH 6 for 10 min in an autoclave at 120°C following overnight incubation with the Aurora A antibody at 4°C in a humid chamber.

Anti-mouse HRP-DAB cell & tissue staining kit (R&D systems; cat. CTS002) was used for antibody detection following the manufacturer’s recommendations. Sections were then counterstained with haematoxylin eosin. The sections were scored positive or negative according to Aurora A nuclear staining. Positive cytoplasmic staining without nuclear staining was defined as negative. The scoring was done by subjective assessment. All sections were scored by the same two individuals in a blinded manner.

**BRCA2 wild type allele loss analysis**

**BRCA2** wild-type allele loss analysis was performed by TaqMan allele-specific quantitative PCR (qPCR), as previously described, on DNA isolated from breast tumours of a subset of 52 **BRCA2** 999del5 mutation carriers available for analysis [29,35]. Briefly, by using a single forward primer and two different reverse primers, one for the wild type allele and another for the **BRCA2** 999del5 allele, and a FAM-labelled TaqMan probe, the average Ct value was determined for duplicate qPCRs separately for the two primer pairs. For a valid qPCR the differences between the two Ct values of the same primer pair were within 5%. When the **BRCA2** wild-type allele proportion was less than 33% of the total of the **BRCA2** 999del5 and wild-type alleles, the sample was defined as having **BRCA2** wild-type allele loss [30]: this equates to loss in more than 50% of the cells.

**Statistical analysis**

Association between categorical variables was examined using either Fisher’s exact test or Chi-square test using the statistical package GraphPad InStat version 3.01 (GraphPad Software, Inc., San Diego, CA, USA). Univariate survival curves were generated using the Kaplan-Meier method and the Log-Rank test was used for comparing them using XLSTAT 2013.4 (Addinsoft, Paris, France). Patients diagnosed with breast cancer were followed from diagnosis of the first breast tumour until death or last date of follow up (30 September 2013). The outcome was breast cancer-specific survival, defined as the time from diagnosis to death from breast cancer, as registered on death certificates. Patients who died of other causes than breast cancer were censored at the time of death. The underlying assumptions for proportionality for the Cox hazards regression were assessed using the cox.zph function in R 2.15.2 (survival package). All p values were two sided and p values

| Table 1. Patient characteristics at baseline: Clinical and pathological parameters for the 391 breast cancer cases analysed in this study |
|---|
| Parameters | Sporadic cases (n = 284) | BRCA2 mutation carriers (n = 107) | p Value* |
| Aurora A | | | |
| Negative | 136 (47.9%) | 40 (37.4%) | |
| Positive | 148 (52.1%) | 67 (62.6%) | 0.07 |
| Age (years) | | | |
| <50 | 147 (51.8%) | 58 (54.2%) | |
| ≥50 | 137 (48.2%) | 49 (45.8%) | 0.73 |
| Tumour size | | | |
| <20 | 124 (46.8%) | 44 (48.9%) | |
| >20 | 141 (53.2%) | 46 (51.1%) | 0.81 |
| Unknown | 19 | 17 | |
| Nodal status | | | |
| Negative | 141 (52.0%) | 46 (45.5%) | |
| Positive | 130 (48.0%) | 55 (54.5%) | 0.29 |
| Unknown | 13 | 6 | |
| Tumour grade | | | |
| 1 | 24 (14.4%) | 5 (6.5%) | |
| 2 | 71 (42.5%) | 37 (48.1%) | 0.20 |
| 3 | 72 (43.1%) | 35 (45.4%) | |
| Unknown | 117 | 30 | |
| ER | | | |
| Negative | 83 (30%) | 31 (29.2%) | |
| Positive | 194 (70%) | 75 (70.8%) | 0.99 |
| Unknown | 7 | 1 | |
| PR | | | |
| Negative | 120 (43%) | 45 (42.5%) | |
| Positive | 159 (57%) | 61 (57.5%) | 0.99 |
| Unknown | 5 | 1 | |
| Ki-67 | | | |
| <14% | 114 (42.1%) | 37 (34.9%) | |
| ≥14% | 157 (57.9%) | 69 (65.1%) | 0.24 |
| Unknown | 13 | 1 | |
| Ploidy | | | |
| Aneuploid | 89 (55.6%) | 39 (52%) | |
| Diploid | 71 (44.4%) | 36 (48%) | 0.67 |
| Unknown | 124 | 32 | |
| Phenotype | | | |
| Non-luminal | 80 (29.9%) | 27 (25.5%) | |
| Luminal A/B | 188 (70.1%) | 79 (74.5%) | 0.45 |
| Unknown | 16 | 1 | |
| Chemotherapy | | | |
| Yes | 57 (57.6%) | 39 (52%) | |
| No | 42 (42.4%) | 36 (48%) | 0.67 |
| Unknown | 8 | 32 | |
| Radiation | | | |
| Yes | 52 (50.5%) | 37 (34.9%) | |
| No | 51 (49.5%) | 69 (65.1%) | 0.24 |
| Unknown | 13 | 1 | |
| Endocrine treatment (ER positive) | | | |
| Yes | 35 (52.2%) | 27 (25.5%) | |
| No | 32 (47.8%) | 49 (45.4%) | |
| Unknown | 8 | 17 | |

* p Values were from Fisher and Chi-square tests.
Results

Aurora A nuclear expression in breast tumours is associated with poor prognosis

Characteristics of the tumours and treatment type are given in Table 1. In summary, the tumours from sporadic cases and BRCA2 mutation carriers did not differ in size, nodal status, tumour grade, hormone status, Ki-67 expression, ploidy or tumour subgroups (Table 1). Aurora A nuclear expression (Figure 1A) was associated with reduced breast cancer-specific survival in both sporadic and familial BRCA2 breast cancer cases (log-rank $p < 0.001$ and $p = 0.017$, respectively; Figure 1B). For sporadic breast cancer cases with Aurora A nuclear expression, the 10-year survival rate was 70.4% (95% confidence interval [CI] = 64.8 to 75.3%), whereas for BRCA2 mutation carriers it was 53.0% (95% CI = 46.1 to 59.4%). In multivariate survival analysis, the hazards ratios (HR) for breast cancer-specific death associated with Aurora A nuclear expression (Table 2) were 2.74 in non-carriers (HR = 2.74; 95% CI = 1.13 to 6.64; $p = 0.026$) and 6.70 in BRCA2 mutation carriers (HR = 6.70; 95% CI = 1.23 to 36.4; $p = 0.028$). Aurora A nuclear expression still remained as an independent prognostic marker among the BRCA2 mutation carriers when adjuvant treatment including chemotherapy, endocrine therapy or radiotherapy were included in the multivariate Cox’s proportion regression model (HR = 7.06; 95% CI = 1.23–40.6; $p = 0.028$; Table 3).

Poor prognosis is associated with BRCA2 wild type allele loss in combination with Aurora A nuclear staining

BRCA2 mutation carriers with BRCA2 wild-type allele loss in the tumour had significantly lower 10-year breast cancer-specific survival (42.8%; 95% CI = 23.8–61.8) compared with those without BRCA2 wild type allele loss (77.4%; 95% CI = 59.8 – 95.0,

Figure 1. Aurora A nuclear expression in breast tumours in relation to prognosis. (A) Aurora A nuclear expression was scored negative based on no immunohistochemical nuclear staining (left panel) or positive based on brown peroxidase nuclear staining (right panel). Scale bars = 100 μm. (B) Kaplan-Meier estimates of breast cancer-specific survival according to Aurora A nuclear staining in 284 sporadic tumours (left panel) and 107 tumours from BRCA2 mutation carriers (right panel). All statistical tests were two sided.
log rank $p = 0.008$; Figure 2A). About half of the BRCA2 tumours (52%) had lost their wild-type BRCA2 allele. Among the tumour subset with BRCA2 wild-type loss, 67% were also positive for Aurora A nuclear staining compared to 88% of tumours without BRCA2 wild-type allele loss. The 10-year breast cancer-specific survival rate among BRCA2 mutation carrier patients with tumours having both BRCA2 wild-type allele loss and Aurora A nuclear staining was significantly lower (24.1%; 95% CI 19.3–29.1%) compared with the subgroups displaying either only BRCA2 wild-type loss (74.2%; 95% CI 56.2–85.7%) or Aurora A nuclear expression only (77.8%; 95% CI 47.7–91.8%; $p < 0.001$; Figure 2B). The three patients with neither BRCA2 wild-type loss nor Aurora A nuclear staining were alive after 10 years follow-up time. Of the 18 individuals with both BRCA2 wild-type allele loss and positive Aurora A nuclear staining, 16 received adjuvant chemotherapy, endocrine therapy or radiotherapy. After adjusting for treatment and clinical parameters in a multivariate Cox’s proportional hazards regression model, both Aurora A nuclear expression and BRCA2 wild-type allele loss remained independently significant predictors of reduced time to breast cancer-specific death (Table 4). In this model, the HR for positive Aurora A nuclear staining was 10.09 (HR = 10.09; 95% CI = 1.19 – 85.4, $p = 0.034$), whereas the HR of having BRCA2 wild-type allele loss was 9.63 (HR = 9.63; 95% CI = 1.81 – 51.0, $p = 0.008$).

**Discussion**

In the present study, we show that Aurora A nuclear expression in breast tumour tissue predicts significantly worse breast cancer-specific survival among both BRCA2 mutation carriers and sporadic cases. For both the groups, Aurora A outperforms other known prognostic markers such as Ki-67. This is in agreement with a study based on more than 3000 tumour samples from women with breast cancer where Aurora A emerged as the marker of the greatest prognostic significance among ER positive tumours, outperforming other markers including Ki-67 [26]. Aurora A has recently been ranked among the top individual genes in terms of their concordance index values with respect to gene expression and survival data in computational modelling of disease prognosis in breast cancer [36]. There are indications of oncogenic transformation activity of Aurora A with shifts from cytoplasmic staining in non-malignant adjacent breast tissue to both cytoplasmic and nuclear compartments in tumour tissue,
suggesting an oncogenic role for nuclear accumulation [37,38]. Activated phosphorylated Aurora A Thr288 has recently been associated with tamoxifen resistance in ER-positive breast cancer by phosphorylation of ERα at positions Ser167 and Ser305, leading to shorter recurrence-free survival [39]. Another recent study suggests that Aurora A induces endocrine resistance through downregulation of ERα expression [40]. Association of Aurora A kinase with activation of the epithelial-to-mesenchymal transition pathway has been suggested in the development of distant metastases in ER-positive breast cancer [41]. Our recent population study on the Icelandic BRCA2 breast cancer cohort showed that positive ER status was significantly associated with increased breast cancer mortality [33] and we have some indication that this could be Aurora A dependent (supplementary material Figure 1). Several studies have found significant prognostic correlation between Aurora A overexpression and ER positivity in breast tumours [20,21,26]. Moreover, recently it has been shown that sensitivity of cancer cells to chemotherapy and radiotherapy is inversely controlled by Aurora A and BRCA2 through the ATM and Chk2 mediated DNA repair networks [27]. The same study also showed how BRCA2 expression induced apoptosis in Aurora A overexpressing cells treated with cisplatin.

Poor breast cancer-specific survival was found to be strongly associated with a combination of BRCA2 wild-type allele loss and Aurora A nuclear staining in tumours from BRCA2 mutation carriers. BRCA2 mutation carriers with either only BRCA2 wild-type allele loss or only Aurora A nuclear staining in tumours had similar breast cancer-specific survival to sporadic cases with Aurora A nuclear expression. Only three tumours of 52 with a hereditary BRCA2 mutation had neither BRCA2 wild-type allele loss nor Aurora A nuclear expression, suggesting that either of the two might be needed for BRCA2 related breast cancer formation. Since Aurora A and BRCA2 have been shown to negatively interact [27,42], the tumorigenic effect of Aurora A amplification and overexpression may be strong in a BRCA2 heterozygous background.

BRCA2 breast cancer patients with tumours displaying Aurora A nuclear staining have a significantly lowered prognosis even when treated with standard adjuvant chemotherapy, endocrine therapy or radiotherapy. Therefore, treatment that targets Aurora A overexpression may be critical for BRCA2 breast cancer cases, especially when occurring in combination with BRCA2 wild-type allele loss. Our earlier study with the pan Aurora inhibitor ZM447439 showed that BRCA2 999del5 heterozygous cell lines exhibited extensive sensitivity [43]. A novel selective Aurora A inhibitor, MLN8237, which is currently undergoing clinical evaluation, has been shown to cooperate with tamoxifen in cell culture by inhibiting tamoxifen-resistant breast cancer cell survival and tumour growth [39]. Similarly, the same Aurora A inhibitor enhances activity against human breast cancer cells in concurrence with other
chemotherapeutic agents and, thus, may result in synergistic benefits [44,45]. Another possible way of targeting Aurora A overexpressing cancer cells is through PARP inhibition. Aurora A overexpression has been shown to confer sensitivity to PARP inhibition in a BRCA2 heterozygous background by suppressing the response to DNA double-strand breaks [28]. Selective Aurora A and PARP inhibitors are presently being studied in preclinical and early clinical trials. These inhibitors might improve treatment benefits for BRCA2 breast cancer patients overexpressing Aurora A in the future. Therefore, screening for Aurora A nuclear expression should be considered for routine use as a clinical marker for breast cancer, at least in the case of BRCA2 mutation carriers.

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Author contributions

MA, STR and SKB carried out experiments and data analysis; JEE and SKB designed the study; MA, OAS and SKB carried out the statistical analysis; OAS, JGI, AS and LT carried out acquisition of clinical parameters and treatment; MA and SKB drafted the manuscript. All authors read and approved the final manuscript.

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Table 4. Multivariate survival analysis for Aurora A nuclear expression and BRCA2 wild-type allele loss in BRCA2 999del5 breast cancer cases with adjustment for treatment and clinical parameters

| BRCA2 mutation carriers (n = 45) | HR     | 95% CI       | p Value |
|---------------------------------|--------|--------------|---------|
| Aurora A nuclear staining       | 10.09  | 1.19–85.44   | 0.034†  |
| BRCA2 wild-type allele loss     | 9.63   | 1.82–51.02   | 0.008†  |
| ER (pos)                        | 1.53   | 0.28–8.43    | 0.626   |
| Ki-67 (pos)                     | 0.19   | 0.28–8.43    | 0.050   |
| Age at diagnosis                | 1.02   | 0.96–1.08    | 0.590   |
| Year of diagnosis               | 1.01   | 0.91–1.12    | 0.873   |
| Tumour size (T2 vs T1)          | 0.86   | 0.18–4.01    | 0.849   |
| Tumour size (T3 vs T1)          | 4.43   | 0.66–29.77   | 0.126   |
| Nodal status (pos)              | 0.70   | 0.05–8.96    | 0.783   |
| Chemotherapy                    | 0.26   | 0.03–2.35    | 0.230   |
| Endocrine treatment             | 0.60   | 0.09–3.95    | 0.599   |
| Radiation                       | 0.91   | 0.26–3.21    | 0.883   |
| Model score (log rank test) p   | 0.004† |              |         |

*p value < 0.05.
†p value < 0.01.
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SUPPLEMENTARY MATERIAL ON THE INTERNET

Additional Supporting Information may be found in the online version of this article.

Supplementary Figure 1. Kaplan-Meier estimates of breast cancer-specific survival according to Aurora A nuclear staining and oestrogen receptor (ER) expression in tumours of 106 BRCA2 mutation carriers.