Aurora kinase A outperforms Ki67 as a prognostic marker in ER-positive breast cancer

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BACKGROUND: Proliferation has emerged as a major prognostic factor in luminal breast cancer. The immunohistochemical (IHC) proliferation marker Ki67 has been most extensively investigated but has not gained widespread clinical acceptance.

METHODS: We have conducted a head-to-head comparison of a panel of proliferation markers, including Ki67. Our aim was to establish the marker of the greatest prognostic utility. Tumour samples from 3093 women with breast cancer were constructed as tissue microarrays. We used IHC to detect expression of mini-chromosome maintenance protein 2, Ki67, aurora kinase A (AURKA), polo-like kinase 1, geminin and phospho-histone H3. We used a Cox proportional-hazards model to investigate the association with 10-year breast cancer-specific survival (BCSS). Missing values were resolved using multiple imputation.

RESULTS: The prognostic significance of proliferation was limited to oestrogen receptor (ER)-positive breast cancer. Aurora kinase A emerged as the marker of the greatest prognostic significance in a multivariate model adjusted for the standard clinical and molecular covariates (hazard ratio 1.3; 95% confidence interval 1.1–1.5; P = 0.005), outperforming all other markers including Ki67.

CONCLUSION: Aurora kinase A outperforms other proliferation markers as an independent predictor of BCSS in ER-positive breast cancer. It has the potential for use in routine clinical practice.

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The intrinsic molecular subtypes have become central to breast cancer research (Perou et al, 2000; Sorlie et al, 2001). However, their successful translation into clinical diagnostic assays has not yet been achieved and remains a priority if patients are to benefit from the knowledge of the molecular heterogeneity of breast cancer. The current assessment of the histological and clinical characteristics of tumours fails to identify patients most appropriate for adjuvant systemic therapy. Although adjuvant therapy significantly improves breast cancer survival (EBCTCG, 1998; Berry et al, 2005), it is generally accepted that a substantial proportion of patients who are at low risk of relapse are nonetheless receiving adjuvant chemotherapy, hence experience the side effects of the treatment without deriving much benefit (EBCTCG, 1998; Berry et al, 2006). The translation of the intrinsic subtypes of breast cancer into clinical assays may enable us to stratify patients by their likelihood of benefiting from adjuvant treatment.

This problem is most serious amongst patients with oestrogen receptor (ER)+ disease because those with ER− disease are known to derive greater absolute benefit of adjuvant chemotherapy (EBCTCG, 1998; Berry et al, 2006). Indeed, ER has been proposed as a determinant of whether patients should receive chemotherapy (Henderson, 2010; Pritchard, 2011; Regierer et al, 2011), however, according to the largest meta-analysis, the proportional risk reduction for mortality is not significantly different by ER status (EBCTCG, 1998). By gene expression profiling ER+ tumours are classified as luminal A or luminal B (Perou et al, 2000). Luminal B tumours are defined by the expression of higher levels of proliferation-related genes, including MKI67, than luminal A tumours (Perou et al, 2000). Although a proportion of luminal B tumours can be distinguished from luminal A tumours by detecting amplification of human epidermal growth-factor receptor 2 (HER2), the remainder are more difficult to identify. Ki67 expression by immunohistochemistry (IHC) has been used as a means of identifying HER2-negative luminal B tumours, successfully defining a subset of ER+ cases with poor outcome (Cheang et al, 2009). In this case, Ki67 was used as a surrogate tissue-based readout of proliferation in order to recapitulate the classification originally based on clustering of tumour transcriptomes (Perou et al, 2000; Cheang et al, 2009). That proliferation is a powerful prognostic factor in breast cancer is evidenced by its inclusion in the assessment of histological grade as mitotic count, which has recently been shown to be largely responsible for the prognostic value of tumour grade (Abdel-Fatah et al, 2010). Moreover, the prognostic power of multigene predictors in breast cancer has been shown to be almost exclusively attributable to proliferation and cell-cycle-related genes and limited to ER+ breast cancer, because ER− cases are nearly always deemed high risk (Teschendorff et al, 2006; Desmedt et al, 2008; Wirapati et al, 2008).
Although MKI67 is invariably included amongst the proliferation genes of multigene predictors, there are also other cell-cycle-related genes, which have received less attention (Paik et al, 2004; Paik, 2007). Assessment of Ki67 expression by IHC holds promise as a prognostic and predictive biomarker, however, reports have been conflicting and comparison between studies made difficult by varying methodologies and cut-points for positivity (Urruticoechea et al, 2005; Yerushalmi et al, 2010), indeed guidelines have been produced in order to address these limitations (Dowsett et al, 2011). Although Ki67 is not generally used in the routine management of breast cancer, it has recently been recommended by the St Gallen consensus committee for discriminating between luminal A and luminal B tumours (Goldhirsch et al, 2011). Alternative IHC markers of proliferation have been proposed and have included those involved in cell-cycle control, including cyclin E (CCNE1) (Keyomarsi et al, 2002) and those that carry out the function of DNA licensing for replication (Gonzalez et al, 2004, 2005). Both mini-chromosome maintenance protein 2 (MCM2) and geminin (GMNN), which licence DNA for replication and inhibit re-replication of DNA, respectively, have been shown to carry prognostic value in breast cancer (Gonzalez et al, 2003, 2004). Assessment of a panel of cell-cycle-related proteins, including MCM2, has been proposed to differentiate actively cycling cells, those in-cycle but with arrested progression and those out of cycle, which may provide prognostic information in breast cancer (Loddo et al, 2009). Thus in a manner analogous to gene signatures of proliferation, measuring multiple proliferation-related proteins has been hypothesised to carry greater prognostic information than relying on a single marker.

We compared the prognostic value of a panel of proliferation markers measured using IHC in a large cohort of tumours represented in tissue microarrays (TMAs). We selected MCM2, Ki67, aurora kinase A (AURKA), polo-like kinase 1 (PLK1), GMNN and phospho-histone H3 (PHH3) based on their differential expression in the phases of cell cycle (Loddo et al, 2009). Our aims were to establish the marker of greatest prognostic utility and to investigate whether a multi-marker assessment of proliferation offered additional prognostic value compared with a single marker.

MATERIALS AND METHODS

Study population

The prospective population-based study SEARCH (studies of epidemiology and risk factors in cancer heredity) was used for this work. This study primarily includes women aged <70 years with early breast cancer who are identified through the East Anglia Cancer Registry. Details of this study have been published previously (Lesueur et al, 2005). A total of 3093 patients were included. The characteristics of the study cohort are detailed in Table 1. Available data included breast cancer-specific mortality, clinical and treatment data. Previously generated data on the IHC markers ER, progesterone receptor (PR), HER2, cytokeratin 5/6 (CK5/6) and epidermal growth-factor receptor (EGFR) were also available (Blows et al, 2010). The SEARCH study is approved by the Cambridgeshire 4 Research Ethics Committee; all the study participants provided written informed consent.

Tissue microarrays, IHC and scoring

Each tumour was represented by a single 0.6-mm tissue core in a TMA constructed from paraffin-embedded tissue blocks guided by haematoxylin and eosin stained slides marked for invasive carcinoma, as previously described (Kononen et al, 1998). Tissue microarray sections of 3–4 μm thickness were dewaxed in xylene and rehydrated throughgraded alcohols. Immunohistochemistry was conducted using a BondMax Autoimmunostainer (Leica, Bucks, UK). Details of reagents and antigen retrieval conditions are summarised in Supplementary Table S1. Bound primary antibody was detected using a BOND polymer detection kit (Leica) and developed with 3-3′ diaminobenzidine. Slides were inspected for uniformity of staining or assay failure and those not considered interpretable were excluded from assessment. The Ariol platform (Genetix Limited, Hampshire, UK) was used to scan slides and the resulting images were used for scoring. Details of scoring systems for all markers are provided in Supplementary Table S1. Proliferation markers were scored according to the proportion of positive cells only, using an Allred proportion score (0 = 0%, 1 = <1%, 2 = 1–10%, 3 = 11–33%, 4 = 34–66% and 5 = >66%). For MCM2, Ki67, GMNN and PHH3, a cell was considered positive if there was any nuclear signal above background, whereas for AURKA and PLK1, any cell with nuclear or cytoplasmic signal above background was deemed positive (Figure 1).

| Variable | Categories | Number | Percent |
|----------|------------|--------|---------|
| Age at diagnosis | <55 | 1977 | 64 |
| | > 55 | 1116 | 36 |
| | Missing | 0 | 0 |
| Grade | 1 | 610 | 20 |
| | 2 | 1290 | 42 |
| | 3 | 793 | 26 |
| | Missing | 400 | 13 |
| Node status | Negative | 1737 | 56 |
| | Positive | 1067 | 35 |
| | Missing | 289 | 9 |
| Tumour size | <2 cm | 1672 | 54 |
| | 2-4.9 cm | 1143 | 37 |
| | ≥5 cm | 101 | 3 |
| | Missing | 177 | 6 |
| ER status | Negative | 588 | 19 |
| | Positive | 1772 | 57 |
| | Missing | 733 | 24 |
| PR status | Negative | 670 | 22 |
| | Positive | 1692 | 55 |
| | Missing | 731 | 24 |
| HER2 status | Negative | 1973 | 64 |
| | Positive | 272 | 9 |
| | Missing | 848 | 27 |
| Chemotherapy | No | 2067 | 67 |
| | Yes | 1025 | 33 |
| | Missing | 1 | <1 |
| Endocrine therapy | No | 548 | 18 |
| | Yes | 2545 | 82 |
| | Missing | 0 | 0 |

Abbreviations: ER = oestrogen receptor; HER2 = human epidermal growth-factor receptor 2; PR = progesterone receptor.
Definition of molecular subtype

In order to investigate the relationship between proliferation markers and molecular subtype, a surrogate IHC-based classifier was used, as previously described (Blows et al, 2010). Molecular subtypes were defined as: luminal1a (ER+ or PR+, HER2−, CK5/6− and EGFR−), luminal1b (ER+ or PR+, HER2+, CK5/6+ or EGFR+), luminal2 (ER+ or PR+, HER2), HER2 (ER− and PR− and HER2+), core-based phenotype (ER− and PR−, HER2−, CK5/6+ or EGFR+) and five-marker negative phenotype (ER−, PR−, HER2−, CK5/6− and EGFR−).

Statistical analyses

All the analyses were stratified according to the ER status in order to account for the fundamental differences between ER+ and ER− tumours (Pharoah and Caldas, 2010). Correlations between ordinal variables were made using Spearman’s rank correlation coefficient. A log-rank test was used to compare survival between strata in Kaplan–Meier survival plots. Association with survival was assessed using a Cox proportional-hazards model with 10-year breast cancer-specific survival (BCSS) as outcome, providing a hazard ratio (HR) and 95% confidence interval (CI) for each variable. The date of study entry rather than date of diagnosis was used to determine time under observation (left-truncation) in order to adjust for unobserved events (Azzato et al, 2009). For the analysis of associations with clinical characteristics, all the proliferation markers were modelled as dichotomous and the significance of associations was tested by Pearson’s Chi-square test or Fisher’s exact test as appropriate. The dichotomous and the significance of associations was tested by correlation coefficient. A log-rank test was used to compare correlation (Spearman’s $r$ between proliferation markers was strongest for Ki67 and MCM2 ($r_o$ 0.59; $P<0.0001$). Correlation between proliferation markers was strongest for Ki67 and MCM2 (Spearman’s $r=0.55; P<0.0001$) in ER+ disease, whereas in ER− disease, it was Ki67 and GMNN that showed the strongest correlation (Spearman’s $r=0.59; P<0.0001$). These weak to moderate correlations between proteins, putatively tracking the same biological process, may be explained by the proportion of cell cycle during which each protein is expressed. The number of cases with higher Allred proportion scores was smaller for proteins expressed for a shorter period of cell cycle (Table 3). For example, MCM2, which is expressed for the longest period during cell cycle of any of the proteins (early and late G1, G2, S and M), was expressed by 11% of cases (after excluding those with missing data) in >66% of cells and 36% of cases in >10% of cells. In contrast, PHH3, which is expressed for the shortest period during cell cycle (M phase only), was expressed by 11% of cases in >10% of cells, with no cases expressing PHH3 in >66% of cells.

Correlations and associations of proliferation markers

All the proliferation markers were significantly correlated with each other and tumour grade in both ER+ and ER− disease (Table 2). In ER+ disease, GMNN was most strongly correlated with grade with a Spearman’s $r$ of 0.31 ($P<0.0001$). In ER− disease, GMNN and Ki67 were most strongly correlated with grade, each with a Spearman’s $r$ of 0.39 ($P<0.0001$). Correlation between proliferation markers was strongest for Ki67 and MCM2 (Spearman’s $r=0.55; P<0.0001$) in ER+ disease, whereas in ER− disease, it was Ki67 and GMNN that showed the strongest correlation (Spearman’s $r=0.59; P<0.0001$).

RESULTS

The characteristics of the study cohort are summarised in Table 1. There were 465 deaths from breast cancer with 416 occurring within 10 years of diagnosis. Excluding cases with missing data, 75% of the cohort was ER+, 72% was PR+ and 12% was HER2+.

Molecular Diagnostics

Figure 1 Photomicrographs of representative immunostaining for all the proliferation markers.

Defining the molecular subtype

The definition of molecular subtype is based on the expression of specific markers, allowing the classification of breast cancer into distinct subtypes.

- **Luminal1a** (ER+ or PR+, HER2−, CK5/6− and EGFR−)
- **Luminal1b** (ER+ or PR+, HER2+, CK5/6+ or EGFR+)
- **Luminal2** (ER+ or PR+, HER2)
- **HER2** (ER− and PR− and HER2+)
- **Core-based phenotype** (ER− and PR−, HER2−, CK5/6+ or EGFR+)
- **Five-marker negative phenotype** (ER−, PR−, HER2−, CK5/6− and EGFR−)

Statistical analyses

The statistical analyses are performed using Cox proportional-hazards models to assess survival outcomes. Correlation coefficients are calculated using Spearman’s rank correlation test to measure the strength and direction of the relationship between proliferation markers. A log-rank test is used to compare survival between different groups.

- **Correlation coefficients**
  - Ki67 and MCM2: $r_o=0.59; P<0.0001$
  - GMNN and Ki67: $r=0.55; P<0.0001$
  - MCM2 and PHH3: $r_o=0.59; P<0.0001$

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Weak to moderate correlations between proteins, putatively tracking the same biological process, may be explained by the proportion of cell cycle during which each protein is expressed. The number of cases with higher Allred proportion scores was smaller for proteins expressed for a shorter period of cell cycle (Table 3). For example, MCM2, which is expressed for the longest period during cell cycle of any of the proteins (early and late G1, G2, S and M), was expressed by 11% of cases (after excluding those with missing data) in >66% of cells and 36% of cases in >10% of cells. In contrast, PHH3, which is expressed for the shortest period during cell cycle (M phase only), was expressed by 11% of cases in >10% of cells, with no cases expressing PHH3 in >66% of cells.

Proliferation markers were associated with adverse clinical characteristics in ER+ disease. Both AURKA and GMNN were significantly associated with positive lymph node status (Table 4). Of the two, AURKA showed the stronger association with 46% of AURKA+ cases being lymph node positive compared with 35% of AURKA− cases ($P<0.001$). All the proliferation markers, except...
PLK1 and PHH3, were significantly associated with HER2 positivity. MCM2 showed the strongest association with 19% of MCM2+ cases being HER2+ compared with just 6% of MCM2− cases (P<0.001). In contrast, in ER− disease, the pattern of association was less clear with some indication of an association with favourable clinical characteristics (Supplementary Table S2). For example, only PLK1 was significantly associated with lymph node status in ER− cases. However, this association was with negative lymph node status with 65% of PLK1+ cases being lymph node negative compared with 72% of AURKA+ cases (P=0.024). Similarly, both AURKA and PLK1 showed a negative association with HER2 positivity. In all, 81% of AURKA− cases were HER2− compared with 72% of AURKA+ cases (P=0.027) and for PLK1, 89% of positive cases were HER2− compared with 76% of negative cases (P=0.037). These findings lend weight to the idea that the clinical and biological significance of proliferation is different between ER+ and ER− tumours.

**Proliferation markers predict poor outcome in ER+ disease only**

Univariate survival analyses revealed an association between all the proliferation markers and poor outcome in ER+ but not in ER− cases (Table 5 and Supplementary Table S3). For ER+ cases, AURKA, GMNN, PHH3 and MCM2 were best modelled as continuous variables. Both MCM2 and GMNN showed a reduction in hazard with time in both complete and imputed data. Ki67 was the only proliferation marker significantly associated with survival in ER− disease, with an association of nominal significance when imputed data was analysed (HR 1.5; 95% CI 1.0–2.1; P=0.032) and a similar point estimate when cases with missing data were excluded (HR 1.3; 95% CI 0.88–1.8; P=0.195). However, in a model adjusted for tumour grade, Ki67 no longer showed an association with survival in imputed data of ER− cases (HR 1.3; 95% CI 0.88–1.9; P=0.200).

Aurora kinase A and GMNN carried prognostic value independent of each other in ER+ disease. The prognostic value of proliferation markers was compared by multivariate analysis including only the proliferation markers as covariates. Both AURKA and GMNN retained independent prognostic significance in the analyses of complete and imputed data (Table 6, Model 1). This finding supports the hypothesis that different markers of proliferation carry distinct prognostic information by better reflecting the phases of cell cycle (Gonzalez et al, 2004, 2005; Williams and Stoebner, 2007; Loddo et al, 2009). Although MCM2 was also retained in the multivariate model of complete data, this association was not recapitulated when the imputed data was analysed. Ki67 did not provide prognostic information independent of all the other proliferation markers. Aurora kinase A carried prognostic information independent of major clinical and molecular characteristics on multivariate analysis of ER+ disease (Table 6, Model 2). There were 88 deaths from breast cancer in the multivariate model of complete data. The increase in relative risk of event was 40% and 30% for complete
**Table 4** Associations of proliferation markers with clinical characteristics in ER-positive disease

| Variable                | ER positive | MCM2 | Ki67 | AURKA | PLK1 | GMNN | PHH3 |
|-------------------------|-------------|------|------|-------|------|------|------|
| Age at diagnosis        |             |      |      |       |      |      |      |
| <55                     | 776 (63)    | 580  | 637  | 564   | 486  | 362  |
| >55                     | 462 (37)    | 0.68 | 395  | 0.312 | 0.938| 0.486|
| P-value                 |             |      |      |       |      |      |      |
| Tumour type             |             |      |      |       |      |      |      |
| Ductal                  | 897 (72)    | 1.6  | 718  | 0.774 | 536  |
| Lobular                 | 219 (18)    | 0.19 | 197  | 0.131 | 0.74 |
| Other                   | 122 (10)    | 0.096| 117  | <0.001| NA   |
| P-value                 |             |      |      |       |      |      |      |
| Grade                   |             |      |      |       |      |      |      |
| 1                       | 316 (29)    | 0.001| 268  | <0.001| 239  |
| 2                       | 601 (55)    | 0.115| 524  | <0.001| 0.125|
| 3                       | 176 (16)    | 0.922| 129  | <0.001| 0.747|
| P-value                 |             |      |      |       |      |      |      |
| Node status             |             |      |      |       |      |      |      |
| Negative                | 723 (63)    | 0.402| 325  | <0.001| 0.252|
| Positive                | 431 (37)    | 0.038| 333  | <0.001| 0.022|
| P-value                 |             |      |      |       |      |      |      |
| Tumour size             |             |      |      |       |      |      |      |
| <2 cm                   | 711 (59)    | 0.209| 898  | 0.095 | 685  |
| ≥2 cm                   | 457 (38)    | 0.117| 373  | <0.001| 0.068|
| P-value                 |             |      |      |       |      |      |      |
| PR status               |             |      |      |       |      |      |      |
| Negative                | 133 (11)    | 0.163| 112  | <0.001| 0.515|
| Positive                | 1076 (89)   | 0.038| 899  | <0.001| 0.618|
| P-value                 |             |      |      |       |      |      |      |
| HER2 status             |             |      |      |       |      |      |      |
| Negative                | 1085 (94)   | 0.001| 907  | <0.001| NA   |
| Positive                | 75 (6)      | 0.774| 79   | <0.001| NA   |

Abbreviations: AURKA = aurora kinase A; ER = oestrogen receptor; GMNN = geminin; HER2 = human epidermal growth-factor receptor 2; MCM2 = mini-chromosome maintenance protein 2; PHH3 = phospho-histone H3; PLK1 = polo-like kinase 1; PR = progesterone receptor. *Fisher's exact test. Note: Percentages have been rounded to the nearest whole number.

**Table 5** Univariate analysis in ER-positive disease

| Variable | Complete case analysis | Multiple imputation (M = 50) |
|----------|------------------------|-----------------------------|
|          | n | HR (95% CI) | P | T (95% CI) | P | n | HR (95% CI) | P | T (95% CI) | P |
| Grade*   | 1560 | 2.3 (1.8–2.9) | <0.001 | NA | 5.7 | 2.8 (1.1–5.1) | <0.001 | 0.3 | 0.3 (0.2–0.4) | 0.004 |
| Tumour size* | 1705 | 2.5 (1.9–3.1) | <0.001 | NA | 2.4 | 1.9 (1.9–2.9) | <0.001 | NA | NA |
| Node Status | 1637 | 3.9 (2.8–5.4) | <0.001 | NA | 3.4 | 2.6 (4.5) | <0.001 | NA | NA |
| Endocrine therapy | 1771 | 0.22 (0.05–0.90) | 0.036 | 2.4 (0.95–5.8) | 0.063 | 0.3 | 0.09 (1.0) | 0.064 | 2.0 | 0.93 (4.4) | 0.074 |
| Chemotherapy | 1771 | 6.2 (2.3–16.8) | <0.001 | 0.52 (0.28–0.98) | 0.044 | 8.6 | 3.6 (2.06) | <0.001 | 0.45 | 0.26 (0.78) | 0.004 |
| PR | 1710 | 0.49 (0.34–0.70) | <0.001 | NA | 0.51 | 0.36 (0.72) | <0.001 | NA | NA |
| HER2 | 1594 | 2.5 (1.7–3.7) | <0.001 | NA | 2.3 | 1.6 (3.4) | <0.001 | NA | NA |
| MCM2* | 1485 | 1.6 (1.2–2.3) | 0.005 | 0.79 (0.64–0.98) | 0.032 | 1.5 | 1.1 (2.0) | 0.008 | 0.83 | 0.69–1.0 | 0.052 |
| Ki67 | 1599 | 1.8 (1.3–2.5) | <0.001 | NA | 1.9 | 1.4 (2.5) | <0.001 | NA | NA |
| AURKA* | 1358 | 1.6 (1.3–1.9) | <0.001 | NA | 1.5 | 1.2 (1.7) | <0.001 | NA | NA |
| PLK1 | 979 | 2.6 (1.3–5.2) | 0.007 | NA | 1.7 | 0.96 (3.0) | 0.071 | NA | NA |
| GMNN* | 1467 | 2.7 (1.6–4.4) | <0.001 | 0.65 (0.47–0.89) | 0.008 | 2.0 | 1.4 (3.0) | <0.001 | 0.74 | 0.58 (9.6) | 0.021 |
| PHH3* | 1012 | 1.5 (1.1–1.9) | 0.004 | NA | 1.3 | 1.1 (1.6) | 0.016 | NA | NA |

Abbreviations: AURKA = aurora kinase A; CI = confidence interval; ER = oestrogen receptor; GMNN = geminin; HER2 = human epidermal growth-factor receptor 2; HR = hazard ratio; MCM2 = mini-chromosome maintenance protein 2; PHH3 = phospho-histone H3; PLK1 = polo-like kinase 1; PR = progesterone receptor; NA = not available. *Modeled as a continuous variable.
Table 6 Multivariate analysis of proliferation markers in ER-positive disease indicating independent prognostic value of AURKA (bold)

| Variable | Complete case analysis | Multiple imputation (M = 50) |
|----------|------------------------|-----------------------------|
|          | n          | HR (95% CI) | P | T (95% CI) | P   | n          | HR (95% CI) | P | T (95% CI) | P   |
| Model 1  | 589        |             |   |            |     | 2237       |             |   |            |     |
| MCM2a    | 0.43       | (0.23–0.82) | <0.01 | 1.6 (1.1–2.4) | 0.024 | 1.3 (1.1–1.6) | <0.001 | NA | 1.3 (0.95–1.8) | 0.23 |
| AURKAa   | 1.6 (1.1–2.2) | 0.010 | NA | 0.51 (0.26–0.99) | 0.047 | 1.8 (1.2–2.7) | <0.001 | 0.75 (0.59–0.96) | 0.023 |
| GMNNa    | 4.0 (1.4–11.7) | 0.011 | NA | 0.51 (0.26–0.99) | 0.047 | 1.8 (1.2–2.7) | <0.001 | 0.75 (0.59–0.96) | 0.023 |
| Model 2  | 884        |             |   |            |     | 2237       |             |   |            |     |
| Gradea   | 1.4 (0.97–2.0) | 0.077 | NA | 0.51 (0.38–0.75) | <0.001 | 1.7 (1.1–2.5) | 0.012 | NA | 1.3 (1.1–1.5) | 0.005 |
| Node status | 2.9 (1.8–4.6) | <0.001 | NA | 0.54 (0.38–0.75) | <0.001 | 1.7 (1.1–2.5) | 0.012 | NA | 1.3 (1.1–1.5) | 0.005 |
| Tumour sizea | 1.6 (1.1–2.4) | 0.018 | NA | 0.54 (0.38–0.75) | <0.001 | 1.7 (1.1–2.5) | 0.012 | NA | 1.3 (1.1–1.5) | 0.005 |
| PR       | 0.51 (0.31–0.85) | 0.010 | NA | 0.54 (0.38–0.75) | <0.001 | 1.7 (1.1–2.5) | 0.012 | NA | 1.3 (1.1–1.5) | 0.005 |
| HER2     | 2.4 (1.4–4.1) | 0.001 | NA | 0.54 (0.38–0.75) | <0.001 | 1.7 (1.1–2.5) | 0.012 | NA | 1.3 (1.1–1.5) | 0.005 |
| AURKAa   | 1.4 (1.1–1.7) | 0.004 | NA | 0.54 (0.38–0.75) | <0.001 | 1.7 (1.1–2.5) | 0.012 | NA | 1.3 (1.1–1.5) | 0.005 |

Abbreviations: AURKA = aurora kinase A; CI = confidence interval; ER = oestrogen receptor; GMNN = geminin; HER2 = human epidermal growth-factor receptor 2; HR = hazard ratio; MCM2 = mini-chromosome maintenance protein 2; PHH3 = phospho-histone H3; PLK1 = polo-like kinase 1; PR = progesterone receptor. *Modelled as a continuous variable.

DISCUSSION

Proliferation has emerged as a robust prognostic factor in ER+ breast cancer (Desmedt et al, 2008; Stuart-Harris et al, 2008; Wirapati et al, 2008). Although mitotic count contributes to tumour grade, additional measures of proliferation have been shown to add prognostic value independent of grade (Alexandarany et al, 2011). Of these, Ki67 labelling by IHC has been most widely investigated (Urruticochea et al, 2005; Cheang et al, 2009; Colozza et al, 2010; Yerushalmi et al, 2010). However, other promising proliferation-related proteins have received less attention as potential prognostic markers (Gonzalez et al, 2003, 2004, 2005; Loddo et al, 2009). We have compared the prognostic utility of a panel of proliferation-related proteins, including Ki67, in a large cohort of primary invasive breast tumours. We confirm that proliferation markers are significantly associated with survival in ER+ disease only and find that AURKA carries the greatest prognostic value outperforming Ki67 and serving as an independent prognostic factor in ER+ breast cancer.

This study has some limitations. First, our conclusions require validation in an independent cohort, even though we have employed a large study cohort (3093 cases) lending statistical robustness to our findings, which are also are in keeping with previous reports (Nadler et al, 2008; Loddo et al, 2009). Second, we have used TMAs to represent tumours. Although excellent concordance between TMAs and full-face sections has been reported (Callagy et al, 2003; Ruiz et al, 2006), further evaluation of AURKA as a clinical assay would require use of full-face sections. Finally, we have not assessed the predictive value of AURKA’s in this observational study, as this would be best addressed in the context of a randomised clinical trial. However, our data support the ability of AURKA to predict absolute benefit of adjuvant systemic therapy, highlighting the potential clinical utility of AURKA.

Prognostic classifiers based on the assessment of tens of genes have followed seminal studies of breast tumour transcriptomes (Perou et al, 2000; Sorlie et al, 2001; Paik et al, 2004; Teschendorff et al, 2006). The prognostic power of these classifiers has been shown to heavily rely on proliferation-related genes (Desmedt et al, 2008; Wirapati et al, 2008). These classifiers utilise several correlated genes to produce a readout of proliferation. Similarly, a panel of IHC proliferation markers has been proposed to show greater prognostic significance than a single marker (Gonzalez et al, 2005; Williams and Stoeber, 2007, 2012; Loddo et al, 2009). The basis of this additional value has been argued to relate to the integration of DNA-licensing markers and markers of actively cycling cells in order to gauge the ‘rate’ of proliferation in a...
given tumour (Gonzalez et al, 2005; Williams and Stoeber, 2007, 2012). The analysis of a panel of cell-cycle-related proteins can identify distinct cell-cycle phenotypes both at the level of single cells (Endl et al, 2001; Shetty et al, 2005) and cell populations (Loddo et al, 2009). Indeed, DNA-licensing factors, particularly MCMs, have been shown to be powerful predictors of clinical outcome in several solid tumours including prostate, lung, kidney, breast and ovary (Meng et al, 2001; Ramnath et al, 2001; Gonzalez et al, 2003, 2004; Dudderidge et al, 2005; Kulkarni et al, 2007). Three cell-cycle phenotypes can be identified by integrating markers of cell-cycle progression, they approximate to (1) an 'out-of-cycle' or differentiated state defined by the lack of expression of cell-cycle proteins, including MCMs, and that may express markers of 'differentiated' cells, including inhibitors of cyclin-dependent kinases such as p27, (2) a 'G1-delayed/ arrested' or growth-arrested state defined by the expression of an MCM, hence DNA is 'licensed' for replication, but lacking expression of mitotic kinases including Plk1 and AURKA or other markers of actively proliferating cells including Ki67 or GMNN and (3) 'accelerated cell cycle' or actively proliferating state defined by the expression of both MCMs and proteins expressed after the cell-cycle restriction point including AURKA, GMNN, PLK1, PHH3 and Ki67 (Endl et al, 2001; Dudderidge et al, 2005; Williams and Stoeber, 2007; Loddo et al, 2009). A scheme for determining cell-cycle phenotype in this way holds particular promise as a predictive biomarker by identifying tumours sensitive to cell-cycle phase-specific chemotherapeutic agents (Williams and Stoeber, 2007, 2012; Loddo et al, 2009). We addressed the hypothesis that multi-parameter estimates of cell-cycle phenotype would outperform single-marker assays as predictors of outcome by including markers expressed differentially during cell cycle in a multivariate analysis. We found that GMNN and AURKA indeed provided independent prognostic information. However, subsequent analysis in a model adjusted for the standard clinical variables showed only AURKA retained independent prognostic value. This may arise as a result of our assessing protein expression as a proportion of a population of cancer cells separately for each cell-cycle marker and subsequently comparing these scores in a multivariate model. This cell-population approach may not identify the proposed cell phenotypes, particularly growth-arrested cells, with adequate sensitivity. A multiplexed single-cell assay, which determines the proportion of cells in each of the three phenotypes per tumour, may overcome this limitation, especially if combined with the sophisticated methods of automated image analysis (Camp et al, 2002; Williams and Stoeber, 2012).

Aurora kinase A is among the proliferation genes that contribute to the 21-gene recurrence score (Paik et al, 2004). Aurora kinase A is required for proper centrosome function and for mitotic spindle assembly (Lens et al, 2010). As a protein, which functions specifically during mitosis, AURKA also represents an attractive drug target and several AURKA inhibitors are under development (Keen and Taylor, 2004; Lens et al, 2010). The basis of the superior prognostic performance of AURKA compared with the other proliferation markers is not clear and is likely to relate to many variables including biological function, assay differences and ease of interpretation. Aurora kinase A was one of the proliferation markers, best modelled as a continuous variable. This is consistent with the idea that luminal tumours form a continuum according to the expression levels of proliferation-related genes and that their division into two subgroups is somewhat arbitrary (Desmedt et al, 2008; Wirapati et al, 2008; Colombo et al, 2011). In this respect, AURKA labelling by IHC could be used as a means of better reflecting this diversity in clinical practice. Moreover, the prognostic utility of AURKA may be increased by including it in a combined index with B-cell lymphoma protein 2, just as we have recently shown for Ki67 (Ali et al, 2012). Moreover, AURKA gene expression has recently been used as a prototypical proliferation marker in a three-classifier model for the molecular subtyping of breast cancer shown to be more statistically robust than other methods (Haiibe-Kains et al, 2012).

In summary, we have conducted a large head-to-head comparison of the prognostic value of a panel of proliferation markers in primary breast cancer. We have used IHC and a scoring system used routinely in clinical practice to show that the prognostic significance of proliferation is limited to ER+ disease and that AURKA outperforms other proliferation markers including Ki67. Aurora kinase A defines five subgroups in ER+ breast cancer and carries independent prognostic significance in multivariate analysis. Our findings show that Ki67 may not be the optimal IHC marker of proliferation and warrant further studies addressing the predictive value of AURKA.

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Figure 3 Bar charts illustrating the relationship between AURKA and (A) grade (B) and molecular subtype in ER-positive disease.
REFERENCES

Abdel-Fatah TM, Pogue DG, Ball G, Lopez-Garcia MA, Habashy HO, Green AR, Reis-Filho JS, Ellis IO (2010) Proposal for a modified grading system based on mitotic index and Bcl2 provides objective determination of clinical outcome for patients with breast cancer. J Pathol 222(4): 388–399

Aleskandarany MA, Rakha EA, Macmillan RD, Pogue DG, Ellis IO, Green AR (2011) MIB1/Ki-67 labelling index can classify grade 2 breast cancer to two clinically distinct subgroups. Breast Cancer Res Treat 127(3): 591–599

Ali AM, Dawson SJ, Blows FM, Provenzano E, Ellis IO, Baglietto L, Huntsman D, Caldas C, Pharoah PD (2011) Comparison of methods for handling missing data on immunohistochemical markers in survival analysis of breast cancer. Br J Cancer 104(4): 693–699

Ali HR, Dawson SJ, Blows FM, Provenzano E, Leung S, Nielsen T, Pharoah PD, Caldas C (2012) A Ki67/BCL2 index based on immunohistochemistry is highly prognostic in ER-positive breast cancer. J Pathol 226(1): 97–107

Azzato E, Greenberg D, Shah M, Blows F, Driver K, Caporaso N, Pharoah P (2009) Prevalent cases in observational studies of cancer survival: do they bias hazard ratio estimates? Br J Cancer 100(11): 1806–1811

Berry DA, Ciurrcinone C, Henderson IC, Citron ML, Budman DR, Goldstein Ali HR, Dawson SJ, Blows FM, Provenzano E, Leung S, Nielsen T, Pharoah PD, Colozza M, Sidoni A, Piccart-Gebhart M (2010) Value of Ki67 in breast cancer. J Natl Cancer Inst 102(22): 1656–1664

Duderdidge TJ, Stoerber K, Loddo M, Atkinson G, Fanshawe T, Grifiths DF, Williams GH (2005) MCM2, Geminin, and Ki67 define proliferative state and are prognostic markers in renal cell carcinoma. Clin Cancer Res 11(7): 2510–2517

EBCTCG (1998) Polychemotherapy for early breast cancer: an overview of the randomised trials. Early Breast Cancer Trialists' Collaborative Group. Lancet 352(9132): 930–942

Endl E, Kausch I, Baack M, Knippers R, Gerdes J, Scholzen T (2001) The expression of Ki-67, MCM3, and p27 defines distinct subsets of proliferating, resting, and differentiated cells. J Pathol 195(4): 457–462

Goldhirsch A, Wood WC, Coates AS, Gelber RD, Thürlimann B, Senn HJ, Members P (2011) Strategies for subtypes—dealing with the diversity of breast cancer: highlights of the St. Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2011. Ann Oncol 22(8): 1736–1747

Gonzalez M, Tachibana K, Chin S, Callagy G, Madine M, Vowler S, Fender S, Laskey R, Coleman N (2004) Geminin predicts adverse clinical outcome in breast cancer by reflecting cell-cycle progression. J Pathol 204(2): 121–130

Gonzalez MA, Pinder SE, Callagy G, Vowler SL, Morris LS, Bird K, Bell JA, Laskey RA, Coleman NA (2003) Minichromosome maintenance protein 2 is a strong independent prognostic marker in breast cancer. J Clin Oncol 21(23): 4306–4313

Gonzalez MA, Tachibana KE, Laskey RA, Coleman N (2005) Control of DNA replication and its potential clinical implication. Nat Rev Cancer 5(2): 135–141

Haible-Kains B, Desmedt C, Loi S, Culhane AC, Bontempi G, Quackenbush J, Sotiriou C (2012) A three-gene model to robustly identify breast cancer molecular subtypes. J Natl Cancer Inst 104(4): 311–325

Henderson JC (2010) Adjuvant chemotherapy is not for everyone. Breast Cancer Res Treat 123(1): 159–162

Hoppin JA, Tolbert PE, Taylor JA, Schroeder JC, Holly EA (2002) Potential for selection bias with tumor tissue retrieval in molecular epidemiology studies. Ann Epidemiol 12(1): 1–6

Keen N, Taylor S (2004) Aurora-kinase inhibitors as anticancer agents. Nat Rev Cancer 4(12): 927–936

Keyomarsi K, Tucker SL, Buchholz TA, Callister M, Ding Y, Hortobagyi GN, Bedrosian I, Knickerbocker C, Toyofuku W, Lowe M, Herlizeck TW, Bacos SS (2002) Cyclin E and survival in patients with breast cancer. N Engl J Med 347(10): 1566–1575

Kononen J, Bubendorf L, Kallioniemi A, Bärlund M, Schraml P, Leighton S, Torhorst J, Mihatsch M, Sauter G, Kallioniemi O (1998) Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat Med 4(7): 844–847

Kukkarni AA, Loddo M, Leo E, Rashid M, Ewald K, Fanshawe TR, Butcher J, Frost A, Ledermann JA, Williams GH, Stoerber K (2007) DNA replication licensing factors and aurora kinases are linked to aneuploidy and clinical outcome in epithelial ovarian carcinoma. Clin Cancer Res 13(20): 6153–6161

Leng SM, Voest EE, Medema RH (2010) Shared and separate functions of polo-like kinases and aurora kinases in cancer. Nat Rev Cancer 10(12): 825–841

Lesueur F, Pharoah P, Laing S, Ahmed S, Jordan C, Smith P, Luben R, Wardham N, Easton D, Dunning A, Ponder B (2005) Allelic association of the human homologue of the mouse modifier Ptpj with breast cancer. Hum Mol Genet 14(16): 2349–2356

Author contributions

HRA, PDP and CC designed the study. HRA scored TMAs and conducted statistical analyses. SJD and EP scored TMAs. FMB constructed TMAs and compiled clinical data. PDP and CC are the project leaders for molecular pathology studies in SEARCH.

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Conflict of interest

The authors declare no conflict of interest.

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J Natl Cancer Inst 100(11): 1806–1811

J Exp Med 175: e1000279

J Natl Cancer Inst 104(4): 311–325

J Natl Cancer Inst 102(22): 1656–1664

J Natl Cancer Inst 100(11): 1806–1811

J Pathol 204(2): 121–130

J Pathol 213(4): 4306–4313

J Cancer website (http://www.nature.com/bjc)
Aurora kinase A outperforms Ki67 as a prognostic marker

Loddo M, Kingsbury SR, Rashid M, Proctor I, Holt C, Young J, El-Sheikh S, Falzon M, Eward KL, Prevost T, Sainsbury R, Stoeker K, Williams GH (2009) Cell-cycle-phase progression analysis identifies unique phenotypes of major prognostic and predictive significance in breast cancer. Br J Cancer 100(6): 959–970

McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM, Diagnostics SSoN-EWGoC (2005) Reporting recommendations for tumor marker prognostic studies (REMARK). J Natl Cancer Inst 97(16): 1180–1184

Meng MV, Grossfeld GD, Williams GH, Dilworth S, Stoeker K, Mulley TW, Weinberg V, Carroll PR, Tlsty TD (2001) Minichromosome maintenance protein 2 expression in prostate: characterization and association with outcome after therapy for cancer. Clin Cancer Res 7(9): 2712–2718

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Regierer AC, Wolters R, Kurzeder C, Wöckel A, Novopashenny I, Possinger K, Wischnewsky MB, Kreienberg R (2011) High estrogen receptor expression in early breast cancer: chemotherapy needed to improve RFS? Breast Cancer Res Treat 128(1): 273–281

Ruz C, Seibt S, Al Kuraya K, Siraj AK, Mirlacher M, Schraml P, Maurer R, Spichkin H, Horsthorst J, Popovska S, Simon R, Sauter G (2006) Tissue microarrays for comparing molecular features with proliferation activity in breast cancer. Int J Cancer 118(9): 2190–2194

Shetty A, Loddo M, Fanshawe T, Prevost AT, Sainsbury R, Williams GH, Stoeker K (2005) DNA replication licensing and cell cycle kinetics of normal and neoplastic breast. Br J Cancer 93(11): 1295–1300

Sørlie T, Perou C, Tibshirani R, Aas T, Johnsen H, Hastie T, Eisen M, van de Rijn M, Jeffrey S, Mesirov J, Brown P, Botstein D, Eyring J, Lonnning P, Børresea-Dale A, Brown P, Botstein D, Eyring J, Lonnning P, Børresea-Dale A (2001) Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci USA 98(19): 10869–10874

Stuart-Harris R, Calsdial S, Pinder SE, Pharoah P (2008) Proliferation markers and survival in early breast cancer: a systematic review and meta-analysis of 85 studies in 32,825 patients. The Breast 17(4): 323–334

Teschendorf AE, Naderi A, Barbosa-Morais NL, Pinder SE, Ellis IO, Aparicio S, Brenton JD, Calsdial S (2006) A consensus prognostic gene expression classifier for ER positive breast cancer. Genome Biol 7(10): R101

Urruticoechea A, Smith IE, Dowsett M (2005) Proliferation marker Ki-67 in early breast cancer. J Clin Oncol 23(28): 7122–7220

Williams GH, Stoeker K (2007) Cell cycle markers in clinical oncology. Curr Opin Cell Biol 19(6): 672–679

Williams GH, Stoeker K (2012) The cell cycle and cancer. J Pathol 226(2): 352–364

Wirapati P, Sorriou C, Kunkel S, Farmer P, Pradervand S, Haibe-Kains B, Desmedt C, Ignatiadis M, Sangtang T, Schutz F, Goldstein DB, Piccart M, Delorenzi M (2008) Meta-analysis of gene expression profiles in breast cancer: toward a unified understanding of breast cancer subtyping and prognosis signatures. Breast Cancer Res 10(4): R65

Yerushalmi R, Woods R, Ravivin D, Hayes M, Gelmon K (2010) Ki67 in breast cancer: prognostic and predictive potential. Lancet Oncol 11(2): 174–183