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Clinical Investigation

MicroRNA-Related DNA Repair/Cell-Cycle Genes Independently Associated With Relapse After Radiation Therapy for Early Breast Cancer

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Summary

Biomarkers of radio-resistance in breast cancer

Purpose: Local recurrence and distant failure after adjuvant radiation therapy for breast cancer remain significant clinical problems, incompletely predicted by conventional clinicopathologic markers. We had previously identified microRNA-139-5p and microRNA-1274a as key regulators of breast cancer radiation response in vitro. The

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are limited. Genes involved in DNA repair and cell cycle control were investigated in independent microarray expression datasets totaling >1000 patients with early-stage breast cancer. Expression of RAD54L, TOP2A, POLQ, RAG1, and SKP2 significantly correlated with local recurrence, survival, or both (multivariate analyses, all $P<.01$). RAD54L, SKP2, and PLK2 may be additionally predictive of response to radiation therapy in vivo. These could select patients for treatment escalation and TOP2A/SKP2 targeted therapies.

purpose of this study was to investigate standard clinicopathologic markers of local recurrence in a contemporary series and to establish whether putative target genes of microRNAs involved in DNA repair and cell cycle control could better predict radiation therapy response in vivo.

Methods and Materials: With institutional ethics board approval, local recurrence was measured in a contemporary, prospectively collected series of 458 patients treated with radiation therapy after breast-conserving surgery. Additionally, independent publicly available mRNA/microRNA microarray expression datasets totaling >1000 early-stage breast cancer patients, treated with adjuvant radiation therapy, with >10 years of follow-up, were analyzed. The expression of putative microRNA target biomarkers—TOP2A, POLQ, RAD54L, SKP2, PLK2, and RAG1—were correlated with standard clinicopathologic variables using 2-sided nonparametric tests, and to local/distant relapse and survival using Kaplan-Meier and Cox regression analysis.

Results: We found a low rate of isolated local recurrence (1.95%) in our modern series, and that few clinicopathologic variables (such as lymphovascular invasion) were significantly predictive. In multiple independent datasets (n>1000), however, high expression of RAD54L, TOP2A, POLQ, and SKP2 significantly correlated with local recurrence, survival, or both in univariate and multivariate analyses ($P<.001$). Low RAG1 expression significantly correlated with local recurrence (multivariate, $P=.008$). Additionally, RAD54L, SKP2, and PLK2 may be predictive, being prognostic in radiation therapy−treated patients but not in untreated matched control individuals (n=107; $P<.05$).

Conclusions: Biomarkers of DNA repair and cell cycle control can identify patients at high risk of treatment failure in those receiving radiation therapy for early breast cancer in independent cohorts. These should be further investigated prospectively, especially TOP2A and SKP2, for which targeted therapies are available.

Introduction

Radiation therapy after breast-conserving surgery (BCS) reduces local and distant recurrence, with metaanalysis showing reduction in the absolute 10-year risk of any recurrence by 15.7% and the 15-year risk of breast cancer death by 3.8% (1). However, local recurrence (LR) and distant failure remain significant clinical problems, incompletely predicted by conventional clinicopathologic markers such as patient age, grade, and lymphovascular invasion.

Biomarkers that predict recurrence after radiation therapy for breast cancer are limited. Molecular subtype appears promising (2, 3), but Ki67 expression has shown contradictory results (4, 5). Scores based on gene expression profiling have been difficult to validate across multiple independent datasets (6, 7). Expression patterns of microRNAs (miRNAs, short noncoding RNAs that downregulate their target genes (8) through posttranscriptional regulation) can characterize cancer phenotypes (9) and are prognostic markers (10-12). High expression of miR-21 (13) and miR-155 (14) have been associated with radioresistance in breast cancer, with the reverse for miR-302 (15), miR-200c (16), and miR-31 (17). The addition of putative target genes can improve the performance of miRNAs as biomarkers with an integrated miRNA−mRNA global profiling approach identifying signatures independently associated with prognosis in breast cancer (12, 18). However, those miRNAs and miRNA targets were investigated in heterogeneous cohorts of patients treated with a mixture of radiation therapy performed after BCS or mastectomy (patient groups with clinically different risk profiles) and treated with and without chemotherapy.

Previous work carried out by our group had identified miR-139-5p conferring radiosensitivity, and miR-1274a radioresistance, along with key target genes, in breast cancer cells in vitro (19). Several of their putative target genes have roles in response to DNA damage: RAD54-like (*Saccharomyces cerevisiae*) (RAD54L), DNA polymerase theta (POLQ), DNA topoisomerase 2-α (TOP2A), recombination activating gene 1 (RAG1), polo-like kinase 2 (PLK2), and cell cycle control: S-phase kinase-associated protein 2 (SKP2). We therefore hypothesized that these genes would be candidate biomarkers of local and distant recurrence after radiation therapy in vivo. To maximize clinical utility, we focused on the group of patients who would be assessed as being at relatively low risk by conventional clinicopathologic markers and treated with adjuvant radiation therapy but not chemotherapy. Early identification of patients within this group with radioresistant tumors would enable escalation of treatment.

In this study, we examined the performance of routine clinicopathologic variables in a series of 458 patients

treated according to modern guidelines. We then built a cohort of >1000 patients with early breast cancer from 3 independent series who were treated with adjuvant radiation therapy (with hormonal therapy as indicated) but no chemotherapy. We interrogated the prognostic utility of putative target genes for local and distant relapse (by univariate and multivariate analysis) and their correlation with clinicopathologic variables. We then investigated the predictive value of these biomarkers in a series of patients from a randomized controlled trial of adjuvant radiation therapy after BCS (6).

Methods and Materials

Patient details: Sydney Series

Patients with early primary breast cancer treated between 1995 and 2013 were prospectively recruited from Royal Prince Alfred Hospital, Sydney Australia. The subset of these patients treated with BCS followed by radiation therapy was analyzed for isolated LR in the ipsilateral breast (same pathology as primary). The patient characteristics are shown in Table 1.

Public database search and patient details for published series

We searched publicly available datasets of patients with early primary breast cancer for whom microarray data (mRNA with or without miRNA), clinicopathologic variables, and at least 10 years of follow-up were available, selecting the subsets of patients who had BCS followed by adjuvant radiation therapy (with hormonal therapy as indicated) but no chemotherapy (Fig. 1). Large subsets of 3 retrospective series, referred to here as Oxford (10, 12), Metabric (20, 21), and NKI (22, 23), were suitable for inclusion (Tables E1A-C; available at www.redjournal.org).

Two case-control series were also analyzed (Tables E1D and E; available at www.redjournal.org). NKI+ (7) consisted of patients from the NKI series with the addition of an independent validation series (119 of 343 experienced LR). The other, here Lund (6), comprised patients, mostly from a randomized clinical trial (24), who experienced local relapse with and without adjuvant radiation therapy after BCS, with case-matched control individuals. Normalized gene expression profiling as publicly deposited was used for analysis. The details of RNA extraction, expression profiling, data extraction, and quality control may be found in Supplementary Methods and Table E2 (available at www.redjournal.org).

Ethics

Written informed consent from each study participant and approval (in accordance with the Helsinki Declaration of 1975, revised 2000) was obtained from the local Institutional Research Ethics Board for the Sydney series and as cited in the individual references for other series. Access to Metabric data was granted through the Data Access Committee of the European Genome-Phenome Archive, and other datasets (NKI, NKI+, and Lund) were downloaded anonymized from the GEO repository (http://www.ncbi.nlm.nih.gov/gds).

Statistical analysis

All statistical analyses were performed with IBM SPSS Statistics version 22. The results were analyzed according to ReMARK recommendations for studies on tumor markers (25). Clinicopathologic correlations were performed with Student t test, analysis of variance, or Spearman rank-order correlation. Endpoints were defined by the STEEP criteria (26) for Institution X and as published for the other datasets. Follow-up was capped once <10 patients were at risk. For univariate Kaplan-Meier analysis, median expression of gene was binary cutoff; differences between subgroups were compared with the log-rank test. For univariate and multivariate Cox regression analysis, gene expression (continuous variable) was ranked and normalized between 0 and 1. $P<.05$ was considered statistically significant, and all tests were 2-sided. Further details of the analysis are given in Supplementary Methods (available at www.redjournal.org).

Results

Isolated LR was rare (n=9, 1.95%) in the prospectively collected series of 458 patients treated with radiation therapy, hormone therapy, and chemotherapy as indicated by contemporary guidelines (Table 1) (Fig. E1; available at www.redjournal.org), similar to other contemporary series (27, 28). With the proviso of very small numbers and short follow-up times, few clinicopathologic variables (such as lymphovascular invasion) previously associated with risk were significantly different between those with and without LR. Thus, novel biomarkers were required to identify these relatively uncommon patients with biologically high-risk disease despite lower-risk pathologic variables.

We assembled a cohort of >1000 patients with early breast cancer from 3 independent retrospective series of early primary breast cancer, with long-term follow-up, expression microarrays, treated without chemotherapy: Oxford, Metabric, and NKI. We also interrogated 2 case-control series: NKI+(enlarged version of NKI selected for LR) and Lund (patients from an adjuvant radiation therapy randomized controlled trial). These series are summarized in Figure 1, and further details are given in the Methods section and in Table E1 (available at www.redjournal.org).

We first considered the univariate association of TOP2A, POLQ, RAD54L, SKP2, PLK2, and RAG1 with local and distant recurrence, because radiation therapy translates into a survival benefit. Local recurrence-free survival (LRFS)

Table 1 Isolated local recurrence after breast-conserving surgery for early breast cancer is poorly predicted by conventional clinicopathologic markers in patients with "modern" treatment

Clinicopathologic characteristic	Local recurrence n=9		No local recurrence n=449	
	Median	Range	Median	Range
Age at diagnosis (y)	56	39-84	54	27-86
Size of primary tumor (mm)	20	3-30	20	3-120
Follow-up time (y)	7.7	3.1-11.5	4.1	0.1-17.1
	Number	%	Number	%
Grade				
Low	1	11.1	79	17.2
Intermediate	2	22.2	192	41.7
High	6	66.7	187	40.7
Unknown	0	0	2	0.4
LVI				
Positive	2	22.2	103	22.4
Negative	7	77.8	321	69.8
Unknown	0	0	25	5.4
Nodal status				
Negative	5	55.6	242	52.6
Positive	4	44.4	174	37.8
Unknown	0	0	33	7.2
Subtype (by IHC)				
Hormone receptor positive	4	44.4	330	71.7
HER2 positive	2	22.2	67	14.6
Triple negative	3	33.3	49	10.7
Unknown	0	0	14	3.0
Stage				
IA	3	33.3	149	32.4
IIA	3	33.3	144	31.3
IIB	2	22.2	79	17.2
IIIA	0	0	34	7.4
IIIC	1	11.1	8	1.7
Characteristic				
Unstageable	0	0	35	7.6
Chemotherapy				
Yes	7	87.5	311	22.5
No	2	25.0	137	77.5
Hormone therapy				
Yes	5	62.5	349	76.0
No	4	50.0	100	21.8
Metastasis				
Yes	2	25.0	37	8.1
No	7	87.5	412	89.8
Death				
All cause	1	11.1	29	6.3
Alive at last review	8	88.9	420	91.5

Abbreviations: IHC = immunohistochemistry; LVI = lymphovascular invasion.
Unable to analyze patients treated without chemotherapy (n=138) as only 2 loca recurrences in this subgroup.

was available for the Oxford and NKI+ series, and recurrence-free survival (RFS) was available for the Oxford series, distant recurrence-free survival (DRFS) for Oxford and NKI, disease-specific survival (DSS) for Oxford and Metabric, and overall survival (OS) for all 3 series.

High expression of RAD54L and TOP2A (putative miR-139-5p targets) were associated with an increased risk of LR in NKI+ (Fig. 2). This was not significant in the Oxford series, perhaps because of fewer events. In Metabric, Oxford, and NKI, high expression of RAD54L, TOP2A, and POLQ was associated with poorer RFS, DRFS, DSS, and OS (Fig. 2A-C) (with trends for RAD54L, Oxford, OS, and TOP2A, NKI, DRFS). The 3 series were also combined together (Total), and all biomarkers were significantly associated with OS.

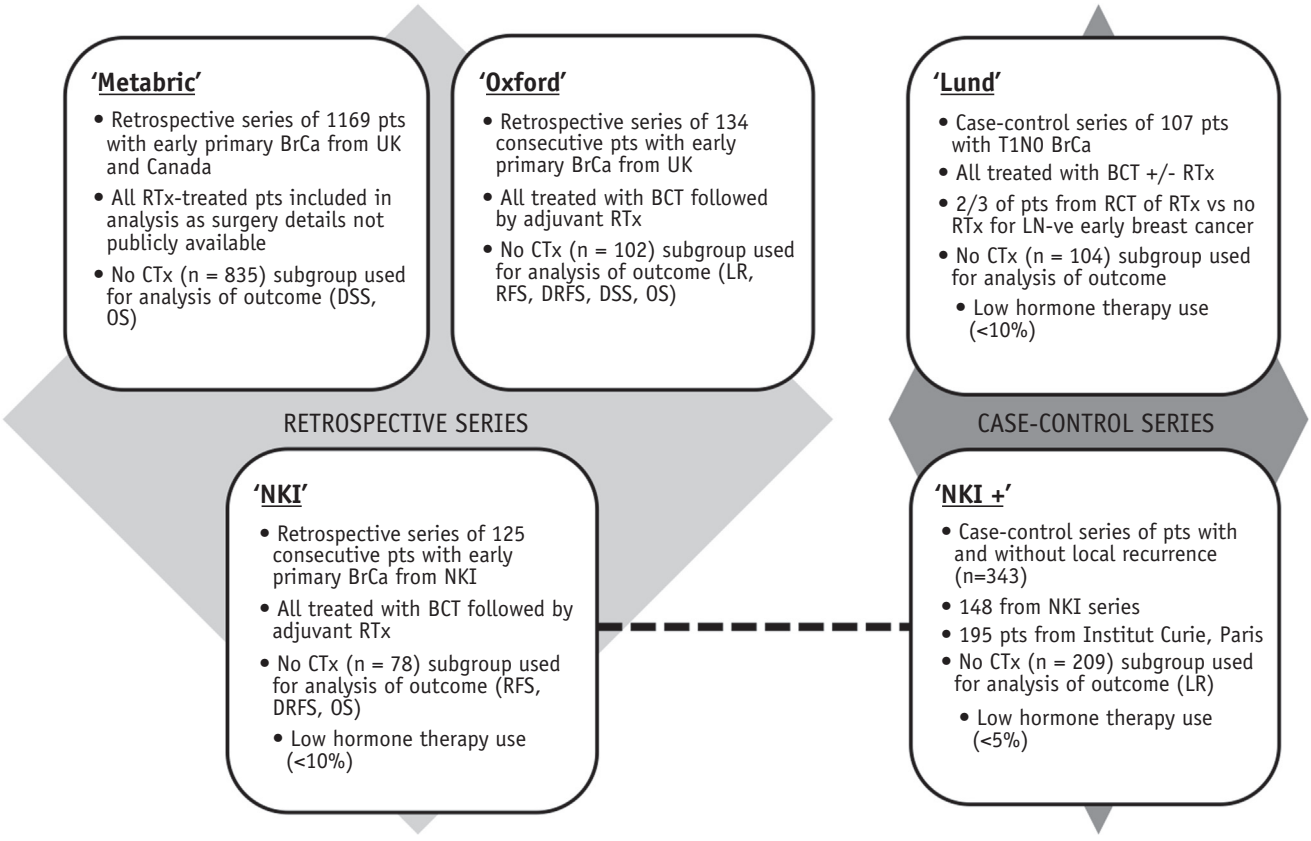

Fig. 1. Details of patient series used in analysis. Subsets were selected from 3 retrospective series and 2 case-control series of patients with early breast cancer. Patients selected had at least 10 years of follow-up, adjuvant radiation therapy (with or without hormonal therapy), but no chemotherapy. There was overlap between the NKI and NKI+ series. *Abbreviations:* +/− = with or or without; BCT = breast conserving surgery; BrCa = breast cancer; CTx = chemotherapy; DRFS = distant recurrence-free survival; DSS = disease-specific survival; LN-ve = lymph node negative; LR = local recurrence; OS = overall survival; pts = patients; RFS = recurrence-free survival; RTx = radiation therapy; UK = United Kingdom.

We next investigated the relationship of SKP2, RAG1, and PLK2 with local and distant recurrence. SKP2 was not associated with LR, but high levels were strongly associated with outcome in both Metabric and NKI series, and with OS in the Total series (Fig. 3A). Lower expression of RAG1 was associated with increased risk of LR in the Oxford series (Fig. 3B) but had no association with other outcomes. PLK2 was more complex. Although it was prognostic in NKI (Fig. 3C), it was prognostic only in the subset of the Oxford series that was estrogen receptor positive (ER+ve, Fig. 3D), and in the Luminal A (ER+ve) subset of Metabric series (log-rank χ^2 4.987, $P=.026$). Owing to insufficient numbers, we did not stratify the series by subtype (>60% were Luminal A and B, as might be expected from a low-risk cohort).

To investigate whether these biomarkers were also predictive of response to radiation therapy, we turned to a case-control series, Lund, with patients selected from a randomized controlled trial of adjuvant radiation therapy after BCS. Patients with LR had been matched to those without LR on clinicopathologic variables including age, tumor size, and grade. Fewer than 10% of these patients were treated with hormonal therapy despite the majority being ER +ve. A probeset for RAG1 was not present on this array.

Elevated levels of TOP2A were associated with LR (Fig. 4A and B) and DSS (Fig. E2; available at www.redjournal.org) whether or not patients received adjuvant radiation therapy. Interestingly, however, elevated levels of RAD54L and SKP2 were significantly associated with LR in the radiation therapy−treated group (Fig. 4C-F) but not in the group without radiation therapy. Similarly, RAD54L and PLK2 were prognostic for DSS only in the radiation therapy−treated group (Figs. E2C-F, available at www.redjournal.org). Notably, although PLK2 was not statistically significantly associated with LR alone, all patients who died of disease had LR first. A test for interaction between RAD54L, SKP2, or PLK2 and radiation therapy was not significant. Taken together, this suggested these biomarkers were predictive of response to radiation therapy.

Many of these genes were associated with clinicopathologic variables that have an impact on outcome. Lower

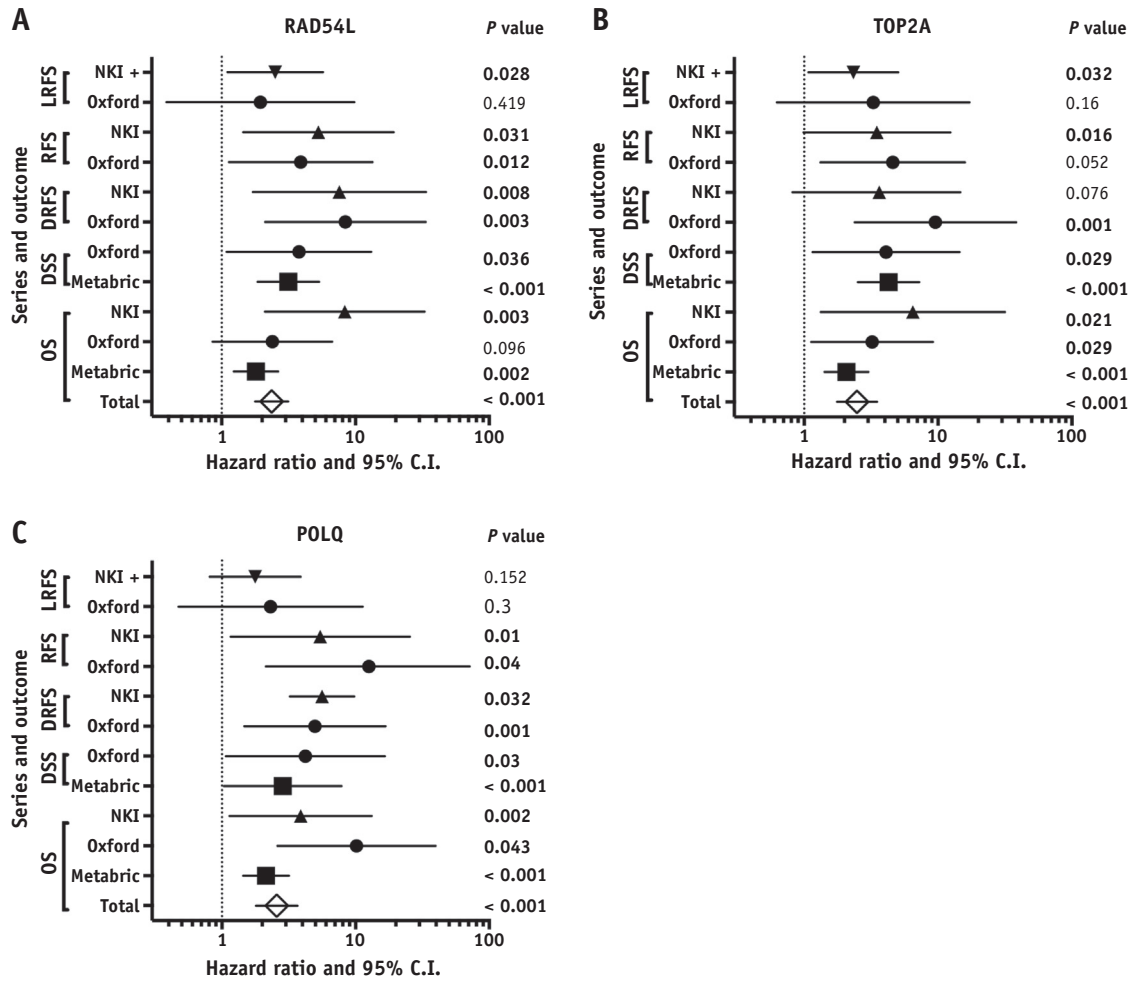

Fig. 2. RAD54L, TOP2A, and POLQ are significantly associated with local and distant recurrence in multiple independent and combined series. Forest plots of hazard ratios (symbols) and 95% confidence intervals (bars) for local recurrence-free survival (LRFS), recurrence-free survival (RFS), distant recurrence-free survival (DRFS), disease-specific survival (DSS) and overall survival (OS) for RAD54L (A), TOP2A (B), and POLQ (C). Univariate Cox proportional hazards model using each biomarker as a continuous ranked variable for individual and combined (Total) series; *P* value for each test given on right-hand side of graph. Symbol dimensions are proportional to dataset size. *Abbreviation:* C.I. = confidence interval.

levels of RAD54L, TOP2A, and POLQ were significantly associated in all 3 series with lower grade, presence of ER expression, and Luminal A subtype, and with age, nodal status, and stage but less consistently across the series (Fig. E3 and Table E3A; available at www.redjournal.org). Lower levels of SKP2 were also significantly associated with markers of improved prognosis, whereas lower levels of PLK2 were significantly associated with markers of adverse prognosis (Fig. E4 and Table E3B; available at www.redjournal.org). RAG1 had few significant associations (Table E3B; available at www.redjournal.org).

We therefore performed multivariate analysis using the genes significant on univariate analysis as continuous ranked variables. The LR multivariate model additionally contained patient age, tumor grade, and molecular subtype (29) (associated with LR on univariate analysis). The survival multivariate model also included ER status, stage, grade, menopausal status, and age (and the individual series itself as a variable if relevant). In the Oxford series, RAG1 remained significantly associated with LR (Fig. 5A); however, none of the biomarkers were independently associated with LR in NKI+. By contrast, elevated expression of RAD54L, TOP2A, POLQ, and SKP2 were independently associated with adverse OS in the combined series (Total: Fig. 5B), DSS in Metabric (Fig. 5C), and DRFS in the Oxford series (Fig. E5; available at www.redjournal.org).

Discussion

Previously, our group performed miRNA microarray profiling on the primary tumors of patients enrolled into a randomized radiation therapy clinical trial (30): 10 who experienced local relapse after BCS and radiation therapy and 10 matched patients who did not. Several differentially

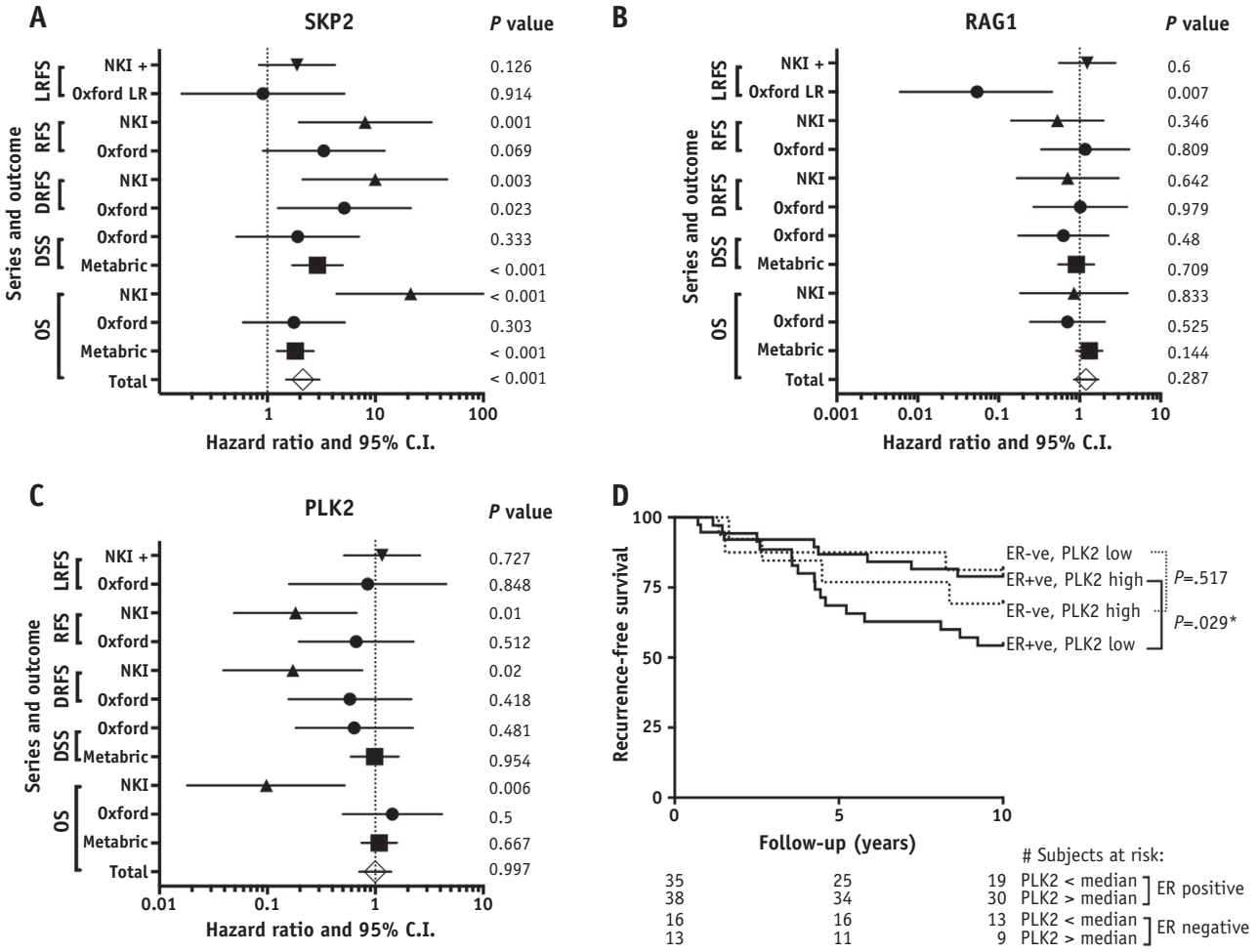

Fig. 3. Association between SKP2, RAG1, and PLK2 and local and distant recurrence in multiple independent and combined series. Forest plots of hazard ratios (symbols) and 95% confidence intervals (bars) for local recurrence-free survival (LRFS), recurrence-free survival (RFS), distant recurrence-free survival (DRFS), disease-specific survival (DSS), and overall survival (OS) for SKP2 (A), RAG1 (B), and PLK2 (C). Univariate Cox proportional hazards model using fractional rank of biomarker expression for individual and combined (Total) series; *P* value for each test given on right-hand side of graph. (D) Low expression of PLK2 is associated with improved recurrence-free survival but only in the estrogen-receptor positive (ER+ve) subset of the Oxford series. *Abbreviation:* C.I. = confidence interval.

expressed miRNAs were identified, of which miR-139-5p and miR-1274a were confirmed functionally in vitro, and miR-139-5p in vivo (19, 31). Putative targets of these miRNAs were predicted using algorithms such as Targetscan and Pictar, and correlations were examined between miRNA and target genes in vivo. Genes with functions important in response to DNA damage (32-34) and cell cycle control (35, 36) several of which have inhibitors either in use or in late-stage development (37-39), were selected for investigation as potential radiation therapy biomarkers (further details in Supplementary Methods; available at www.redjournal.org). (37-39).

To our knowledge, this is the first study investigating putative miRNA target genes as biomarkers of radiation therapy response without including patients treated with chemotherapy. High levels of RAD54L, TOP2A, POLQ, and SKP2 were robustly associated with increased risk of local and distant recurrence across >1000 radiation therapy—treated patients, and RAD54L, SKP2, and PLK2 were also predictive of radio responsiveness. Our hypothesis-driven approach of short-listing targets of radiation therapy—associated miRNAs was reproducible across multiple series, despite the heterogeneity of patients and treatments between cohorts, highlighting these biomarkers' clinical utility.

These genes were shortlisted because current biological understanding suggests possible roles in intrinsic tumor radiosensitivity. RAD54L is a Swi2/Snf2-related translocase with a role in homologous recombination through its association with RAD51 (34, 40, 41). It has been associated with prognosis in several non-breast cancers (42, 43) but not with radiation therapy sensitivity previously in vivo. POLQ can induce radioresistance in vitro (44), and although its overexpression has been shown to confer a

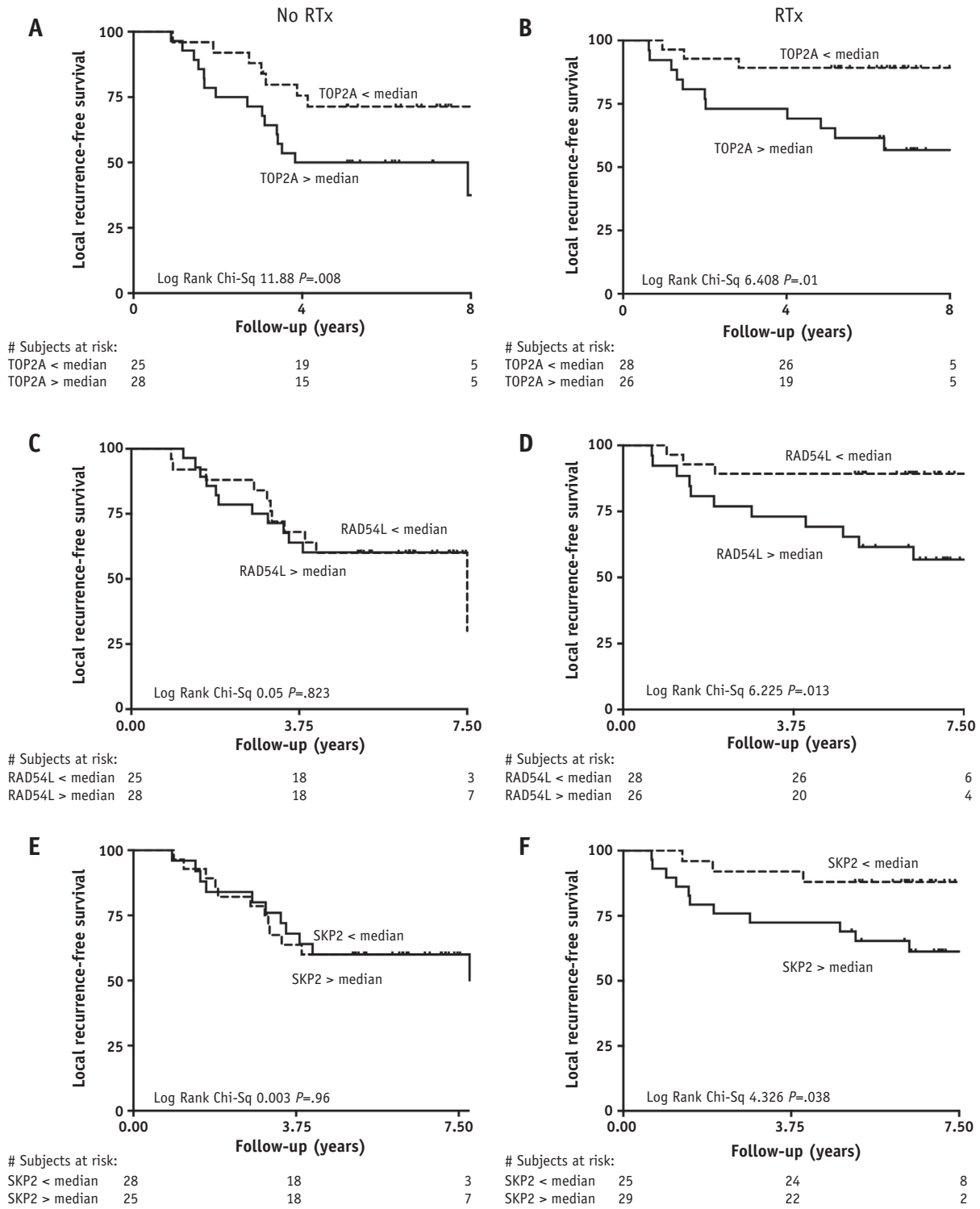

Fig. 4. RAD54L and SKP2 are predictive of local recurrence after radiation therapy and breast conserving therapy (BCT). Kaplan-Meier survival curves for local recurrence-free survival in 107 patients from the Lund case-control series of patients treated with or without adjuvant radiation therapy after BCT. TOP2A is associated with prognosis in both groups (A, B) but RAD54L (C, D) and SKP2 (E, F) are associated only in the radiation therapy−treated group. Series stratified by median expression of each biomarker, log-rank χ^2 and P values indicated. Hazard ratio and 95% confidence interval for each panel: A: 0.438, 0.1959 to 0.964; B, 0.22, 0.09 to 0.73; C, 1.1, 0.48 to 2.5; D, 0.23; 0.091 to 0.75; E, 0.98, 0.42 to 2.26; F, 0.28, 0.11 to 0.93.

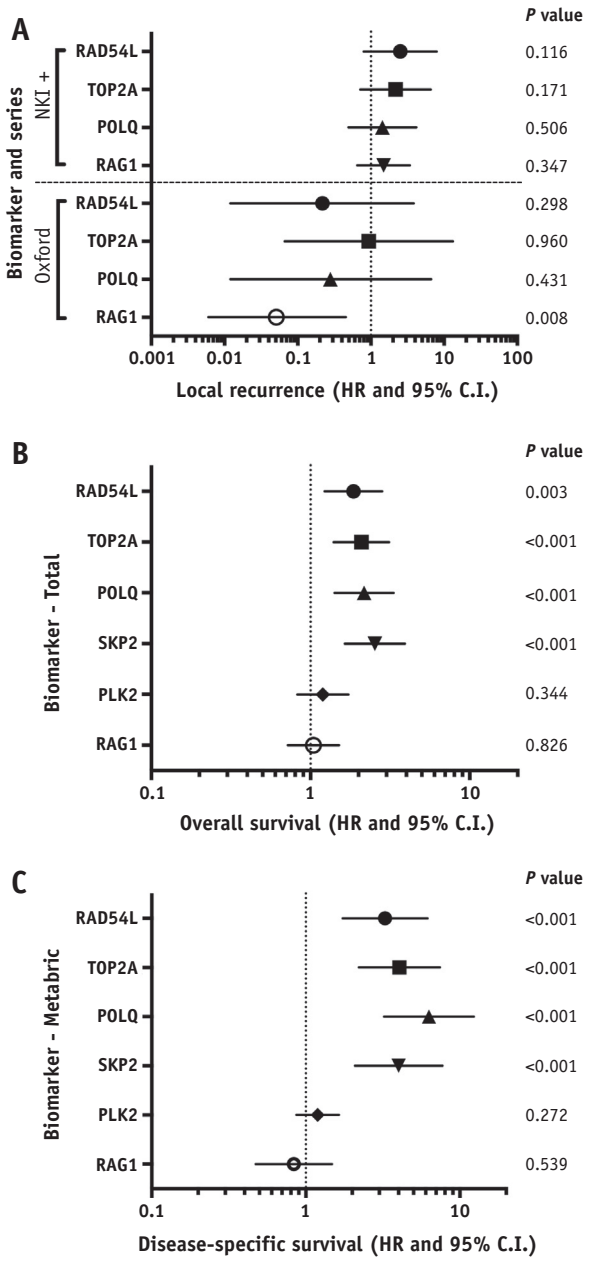

Fig. 5. RAD54L, TOP2A, POLQ, SKP2, and RAG1 are significantly associated with outcome on multivariate analysis. Forest plots of hazard ratios (HR, symbols) and 95% confidence intervals (C.I., bars) for multivariate analysis of outcome. (A) Local recurrence-free survival in NKI+ and Oxford series. (B) Overall survival in the 3 series combined together. (C) Disease-specific survival in the Metabric series. The expression of each biomarker was entered in a Cox proportional hazards model, along with clinicopathologic variables as indicated in text. *P* values on right-hand side of graph.

poor prognosis in breast cancer patients (45), our study confirmed this in a larger cohort of patients without chemotherapy. TOP2A is a well-described marker of proliferation (46) but has not previously been shown to be a marker of prognosis in connection with radiation therapy. SKP2 is a protooncogene required for the ubiquitin-mediated degradation of the CDK inhibitor p27 (47) and its overexpression is associated with radiation resistance in vitro and in vivo in esophageal cancer (48), likely through its role in G1-S checkpoint regulation.

RAG1 and PLK2 were more complex. Although RAG1 was a very strong marker of LR in the Oxford series, this was not the case in the NKI+ series. In both series, the majority of tumors were ER positive, but whereas most received hormonal therapy in the Oxford series, the reverse was true in the NKI+. Second, the overall range of expression of RAG1 was lower in the NKI+ than in the Oxford series (Fig. E6; available online at www.redjournal.org). Interestingly, although the original authors developed a gene classifier for LR in this series, they found that only age remained significant on multivariate analysis (49). PLK2 appeared to have a stronger association with prognosis in ER-positive tumors, and its role in cell cycle control and apoptosis (36) should be further explored in this subtype.

The Lund series, which is unique to our knowledge because radiation therapy after BCS is now standard of care, suggests that RAD54L, SKP2, and PLK2 may be predictive, as well as prognostic, biomarkers. There are several provisos, however: the series is small, and radiation therapy itself was not statistically significantly associated with reduced LR (hazard ratio 0.58, $P = .1$, confidence interval 0.3-1.1). Few patients were treated with hormonal therapy, which decreases any confounding effect but is not modern practice. Thus, although these results are hypothesis generating, further prospective work is required, especially focusing on patients who derive significant absolute benefit from radiation therapy.

Because of the long follow-up times required, in the time period (1984 to 1997) during which these patients were treated, human epidermal growth factor receptor 2 (HER2) status was not measured in the NKI, Lund, or a subset of the Oxford series (34%). No patients were treated with trastuzumab. HER2 overexpression is known to cause radioresistance, and trastuzumab is a radiosensitizer in breast cancer cells (50). However, this is unlikely to have a significant impact on our results: HER2-positive (immunohistochemistry) cases were a minority of the Metabric (5.3%), Oxford (14%), and NKI+ (11%) series, and HER2 molecular subtype was a minority of the cases in all series (2% to 13%), most likely because those patients who had HER2-positive tumors had other high-risk features that caused them to be excluded from our study. Furthermore, with the limitation of the small numbers, HER2 did not have a significant interaction with the biomarkers (univariate or multivariate modeling; data not shown).

Given that overexpression of RAD54L, TOP2A, POLQ, and SKP2 is associated with adverse prognosis, specific inhibitors could be investigated as radiosensitizers. Etoposide and doxorubicin are inhibitors of TOP2A that are

currently used for breast cancer treatment (albeit sequentially). Recently a SKP2 inhibitor was shown to be a growth inhibitor in xenograft models, although the relationship with radiation was not examined (38). POLQ inhibitors are being developed. Thus, these biomarkers could select patients for whom traditional clinicopathologic markers do not suggest a high risk of relapse, but the addition of targeted radiosensitizers would make radiation more effective.

In summary, we found that expression of RAD54L, TOP2A, POLQ, SKP2, and RAG1 were associated with risk of local failure, distant failure, or both, in large independent cohorts of patients with early breast cancer. These biomarkers should be investigated prospectively, particularly for the selection of specific inhibitors.

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