Development and Validation of a Gene Profile Predicting Benefit of Postmastectomy Radiotherapy in Patients with High-Risk Breast Cancer: A Study of Gene Expression in the DBCG82bc Cohort

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Abstract

Purpose: To identify genes predicting benefit of radiotherapy in patients with high-risk breast cancer treated with systemic therapy and randomized to receive or not receive postmastectomy radiotherapy (PMRT).

Experimental Design: The study was based on the Danish Breast Cancer Cooperative Group (DBCG82bc) cohort. Gene-expression analysis was performed in a training set of frozen tumor tissue from 191 patients. Genes were identified through the Lasso method with the endpoint being locoregional recurrence (LRR). A weighted gene-expression index (DBCG-RT profile) was calculated and transferred to quantitative real-time PCR (qRT-PCR) in corresponding formalin-fixed, paraffin-embedded (FFPE) samples, before validation in FFPE from 112 additional patients.

Results: Seven genes were identified, and the derived DBCG-RT profile divided the 191 patients into “high LRR risk” and “low LRR risk” groups. PMRT significantly reduced risk of LRR in “high LRR risk” patients, whereas “low LRR risk” patients showed no additional reduction in LRR rate. Technical transfer of the DBCG-RT profile to FFPE/qRT-PCR was successful, and the predictive impact was successfully validated in another 112 patients.

Conclusions: A DBCG-RT gene profile was identified and validated, identifying patients with very low risk of LRR and no benefit from PMRT. The profile may provide a method to individualize treatment with PMRT. Clin Cancer Res; 20(20); 5272–80. ©2014 AACR.

Introduction

Radiotherapy is known to improve locoregional control and disease-free survival (DFS), and shows a long-term improvement in overall survival (OS) in high-risk patients suffering from breast cancer (1). Postmastectomy radiotherapy (PMRT) is currently administered according to clinicopathologic criteria, defining the patient’s a priori risk of subsequent locoregional recurrence (LRR), and not according to individual prediction of the likelihood of benefit from radiotherapy.

Results from the randomized trials, Danish Breast Cancer Cooperative Group (DBCG) protocol 82bc and the British Columbia Randomized Radiation trial, showed a substantial OS benefit after PMRT even in patients with 1 to 3 positive nodes (2–4), and this has been supported by the Early Breast Cancer Trialists’ Collaborative Group (EBCTCG) 2014 overview (5) as well. Recommendation for PMRT is especially consolidated in patients estimated to have a high risk of LRR (e.g., involvement of ≥4 lymph nodes or tumor size > 5 cm; refs. 6, 7). The most recent St. Gallen Consensus (2011; ref. 8) further supports the administration of PMRT to patients <45 years of age with 1 to 3 positive nodes, as well as to patients estimated to have a high risk of LRR (e.g., involvement of ≥4 lymph nodes or tumor size > 5 cm; refs. 6, 7). The most recent St. Gallen Consensus (2011; ref. 8) further supports the administration of PMRT to patients <45 years of age with 1 to 3 positive nodes, as well as to patients estimated to have a high risk of LRR (e.g., involvement of ≥4 lymph nodes or tumor size > 5 cm; refs. 6, 7). The most recent St. Gallen Consensus (2011; ref. 8) further supports the administration of PMRT to patients <45 years of age with 1 to 3 positive nodes, as well as to patients estimated to have a high risk of LRR (e.g., involvement of ≥4 lymph nodes or tumor size > 5 cm; refs. 6, 7). The most recent St. Gallen Consensus (2011; ref. 8) further supports the administration of PMRT to patients <45 years of age with 1 to 3 positive nodes, as well as to patients estimated to have a high risk of LRR (e.g., involvement of ≥4 lymph nodes or tumor size > 5 cm; refs. 6, 7). The most recent St. Gallen Consensus (2011; ref. 8) further supports the administration of PMRT to patients <45 years of age with 1 to 3 positive nodes, as well as to patients estimated to have a high risk of LRR (e.g., involvement of ≥4 lymph nodes or tumor size > 5 cm; refs. 6, 7). The most recent St. Gallen Consensus (2011; ref. 8) further supports the administration of PMRT to patients <45 years of age with 1 to 3 positive nodes, as well as to patients estimated to have a high risk of LRR (e.g., involvement of ≥4 lymph nodes or tumor size > 5 cm; refs. 6, 7). The most recent St. Gallen Consensus (2011; ref. 8) further supports the administration of PMRT to patients <45 years of age with 1 to 3 positive nodes, as well as to patients estimated to have a high risk of LRR (e.g., involvement of ≥4 lymph nodes or tumor size > 5 cm; refs. 6, 7). The most recent St. Gallen Consensus (2011; ref. 8) further supports the administration of PMRT to patients <45 years of age with 1 to 3 positive nodes, as well as to patients estimated to have a high risk of LRR (e.g., involvement of ≥4 lymph nodes or tumor size > 5 cm; refs. 6, 7). The most recent St. Gallen Consensus (2011; ref. 8) further supports the administration of PMRT to patients <45 years of age with 1 to 3 positive nodes, as well as to patients estimated to have a high risk of LRR (e.g., involvement of ≥4 lymph nodes or tumor size > 5 cm; refs. 6, 7). The most recent St. Gallen Consensus (2011; ref. 8) further supports the administration of PMRT to patients <45 years of age with 1 to 3 positive nodes, as well as to patients estimated to have a high risk of LRR (e.g., involvement of ≥4 lymph nodes or tumor size > 5 cm; refs. 6, 7). The most recent St. Gallen Consensus (2011; ref. 8) further supports the administration of PMRT to patients <45 years of age with 1 to 3 positive nodes, as well as to patients estimated to have a high risk of LRR (e.g., involvement of ≥4 lymph nodes or tumor size > 5 cm; refs. 6, 7). The most recent St. Gallen Consensus (2011; ref. 8) further supports the administration of PMRT to patients <45 years of age with 1 to 3 positive nodes, as well as to patients estimated to have a high risk of LRR (e.g., involvement of ≥4 lymph nodes or tumor size > 5 cm; refs. 6, 7). The most recent St. Gallen Consensus (2011; ref. 8) further supports the administration of PMRT to patients <45 years of age with 1 to 3 positive nodes, as well as to patients estimated to have a high risk of LRR (e.g., involvement of ≥4 lymph nodes or tumor size > 5 cm; refs. 6, 7).
Translational Relevance

Postmastectomy radiotherapy (PMRT) is currently administered according to a clinicopathologic estimation of the patient’s risk of locoregional recurrence (LRR). The presented DBCG-RT gene profile consistently predicts LRR in patients with early breast cancer and predicts benefit from PMRT. The DBCG-RT profile identifies a subgroup of patients with low risk of LRR, who from traditional clinicopathologic risk estimation would be considered to have a high risk of LRR and as such eligible for PMRT. These patients defined by the DBCG-RT profile as having a low risk of LRR and no additional benefit from PMRT in terms of increased local control may potentially be spared the risk of radiation-induced morbidity and secondary cancer.

The DBCG-RT profile works on routinely processed formalin-fixed, paraffin-embedded tissue and is potentially applicable in a daily clinical setting.

With systemic treatment and randomized to receive or not to receive PMRT.

Materials and Methods

Patients

The DBCG82bc cohort has been described in detail elsewhere (2, 3), (21, 22). In brief, 3,083 patients with high-risk breast cancer (<70 years of age) treated with mastectomy and partial axillary dissection were included in the period from 1982 to 1990. All patients were randomized to PMRT. Premenopausal women (DBCG82b) were treated with cyclophosphamide, methotrexate, and fluorouracil (CMF), and postmenopausal women (DBCG82c) were treated with tamoxifen. Radiotherapy was delivered as an anterior photon field against the supraclavicular, infraclavicular, and axillary lymph nodes, and an anterior electron field against the chest wall and intramammary lymph nodes. Intended dose was 50 Gy/25 fractions/5 weeks or 48 Gy/22 fractions/5 1/2 weeks (2, 3, 23). A median of 7 axillary lymph nodes was removed.

Methods

Fresh-frozen tumor (FFT) samples were available from 273 DBCG82bc patients. Extraction of mRNA from FFT and microarray analysis were performed as described in detail in Myhre and colleagues (24). Microarray analysis was successful in 70% (191 of 273) of the frozen samples, containing >5% invasive carcinoma (Supplementary Fig. S1), and these constituted the training set. The microarray data have previously been published and submitted to GEO with accession number GSE24117 (24).

To identify a gene profile that is predictive of benefit from adjuvant PMRT, genes whose expression levels interacted with PMRT on the association with LRR were first identified through a two-step Cox Proportional Hazard model with Lasso penalty (25), and a weighted Cross-Validated Score Index (CVSI), based on the expression levels of the identified genes, was calculated as described in detail in Supplementary Document S1. This was performed in all 191 patients of the training set. The number of patients receiving CMF in the group of patients randomized to PMRT (46 of 97, 47%) was not statistically significant from nonirradiated patients receiving CMF (41 of 94, 44%; P = 0.66). Second, the prognostic value of the gene profile was tested in the subset of 94 of 191 nonirradiated patients.

From the rest of the DBCG82bc cohort, only formalin-fixed, paraffin-embedded (FFPE) samples were available. Therefore, a technical transfer of the identified genes and the derived index to qRT-PCR and FFPE was needed to proceed to validation. The technical transfer was carried out in 146 of 191 patients of the training set, from whom corresponding FFPE samples with >5% invasive carcinomas were available (Supplementary Fig. S1). Details on transfer of technology and recalculation of the index to make it independent of the training set are described in Supplementary Document S2.

The DBCG82bc cohort has previously been criticized for a limited axillary surgical procedure, potentially influencing
the rate of local recurrences in the cohort. Therefore, the validation set was chosen to originate from the subgroup of 1,001 DBCG82bc patients with the most extensive axillary surgery (>7 lymph nodes removed) and with FFPE with histologically verified tumor content available (Supplementary Fig. S2), previously included in the study by Kyndt and colleagues (15). The more extensive surgical procedure in the validation set was expected to be associated with a lower LRR rate in this group of patients.

Extraction of mRNA from FFPE using the Tissue Preparation System together with VERSANT Tissue Preparation Reagents (Siemens Healthcare Diagnostics), and subsequent qRT-PCR using the Fluidigm Biomark 96.96 dynamic gene expression system, preceded by a preamplification step, was carried out as previously described (26–29). Inventoried TaqMan Gene Expression Assays (Applied Biosystems) were used if available (Supplementary Table S2). ER- and HER2-receptor status was available from IHC analyses on tissue micro arrays (30). If IHC values were missing, ER was supplemented with original biochemical analyses (31) retrieved from clinical records (15% = 29/191 patients), and HER2 with gene-expression–derived ERBB2 status (16% = 31 of 191 patients), both known to correlate well to the IHC status (27). All assays were performed blinded to the study endpoint.

Statistical analysis
The endpoint considered was LRR. In agreement with the original publications on the cohort (2, 3), LRR was defined as the appearance of local or regional disease (chestwall, axilla, supra/infracavicular) occurring as an isolated event, or at least 1 month before DM, or simultaneously with DM within ± 1 month. LRR occurring more than 1 month after DM was censored at time to DM, and did not count as an LRR. Patients with DM and no LRR were censored at DM time, and patients with neither DM nor LRR were censored at last date of vital status/follow-up. The date for assessment of recurrence and vital status was January 1, 2012.

The Fisher exact test was used for testing relationship between categorical variables as well as between the CVSI index calculated from FFT and FFPE in the training set. A competing risk model was used for calculating cumulative incidence with inclusion of death before LRR or development of DM as competing events. Cumulative incidence probability curves were plotted and tested for differences (Wald test). Cox uni- and multivariate regression analyses were performed, and assumptions of proportional hazards were tested graphically using log-minus-log plots. Level of significance was 5%, and all estimated P values were two-sided. Statistical calculations were performed using STATA version 11.2 (StataCorp) and R [Development Core Team (2011); ref. 32].

Results
Clinical characteristics and outcome description
In the training set, 53 of 191 patients with FFT available experienced an LRR, and 40 of 146 patients included in the technical transfer experienced an LRR. In the validation set, 20 of 112 patients experienced an LRR. The difference in number of events in the training set and validation set was not significant (P > 0.05). Median follow-up time was 25.1 years for patients in the training set, and 24.6 years for patients in the validation set. The median age of the patients included in the validation set was lower than in the training set (Table 1 and Supplementary Table S1) with a higher fraction of premenopausal women in the validation set (P = 0.006). The validation set further included a higher fraction of patients with small tumors (<2 cm; P = 0.001) and HER2-negative tumors (P < 0.0001), but the distribution of other clinical parameters was similar.

Median tumor area fraction was 50% (range, 5%–85%) in the 191 frozen samples of the training set, 60% in the corresponding 146 FFPE samples (range, 5%–100%), and 60% (range, 5%–100%) in the 112 FFPE samples of the final validation set.

Development of a radiation profile in the training set
From the microarray data, 7 probes whose transcripts interact with PMRT to modify the hazard of LRR were identified (HLA-DQA, RGS1, DNLAI1, hCG2023290, ICKC, OR6G2, and ADH1B). A weighted CVSI index based on the 7 probes was calculated (Supplementary Document S1), and the 191 patients were ranked according to the size of the index. On the basis of a cumulated odds ratio plot, a cutoff was chosen that separated the patients of the training set into two groups: “low LRR risk” (high index) and “high LRR risk” (low index; for details, see Supplementary Document S1). The cutoff coincided with the upper quartile of the index. In both risk prediction groups, the patients were well distributed between the two randomization arms for all evaluated clinicopathologic parameters. A “low LRR risk” group could be identified among all clinicopathologic subgroups, even in subgroups with tumor size >5 cm or ≥4 positive lymph nodes (Table 1).

There was no statistically significant difference in the distribution of clinicopathologic parameters between the two groups, except for ER.

Prognostic impact in the nonirradiated group of patients in the training set
The prognostic value of the gene profile (DBCG-RT profile) was evaluated in the nonirradiated subgroup of the training set. Among the 94 patients treated with systemic treatment alone, the “high LRR risk” patients were shown to have significantly higher risk of LRR as compared with the patients with “low LRR risk” [57% vs. 8% at 20 years; P < 0.0001; unadjusted HR, 0.09; 95% confidence intervals (CI), 0.02–0.36; Fig. 1]. The DBCG-RT profile remained a significant prognostic factor for local failure in a multivariate Cox regression analysis, when adjusting for lymph node status and locally advanced disease (tumor size >5 cm or skin or fascia invasion; P < 0.0001; adjusted HR, 0.07; 95% CI, 0.02–0.30).

Predicting benefit of PMRT in the training set
When analyzing the two risk groups separately in the training set of 191 patients and stratifying them according to
PMRT, a predictive value can be seen in Fig. 2A and B. Radiotherapy significantly improved local control rates in "high LRR risk" patients (57% vs. 12% at 20 years; $P \leq 0.0001$; adjusted HR, 0.17; 95% CI, 0.08–0.34; Fig. 2A), equalizing the rate to "low LRR risk" patients, who showed no additional improvement of local control by PMRT (8% vs. 9% at 20 years; $P = 0.93$; adjusted HR, 1.13; 95% CI, 0.14–9.15; Fig. 2B). Unadjusted HRs can be seen in Table 2. The DBCG-RT profile identified a subset of patients with a "low LRR risk" profile even among subgroups traditionally considered to have a high risk of recurrence, and benefit from PMRT was, on the other hand, found for "high LRR risk" patients even when having small tumors (<2 cm; Supplementary Fig. S3A) or 1 to 3 positive lymph nodes.

### Table 1. Distribution of clinicopathologic parameters among 191 patients included in the training set, randomly assigned to receive PMRT or not receive PMRT (No PMRT), and stratified according to the DBCG-RT profile ("low LRR risk" vs. "high LRR risk") determined from microarray analysis based on FFT

<table>
<thead>
<tr>
<th>Training set</th>
<th>No PMRT</th>
<th>PMRT</th>
</tr>
</thead>
<tbody>
<tr>
<td>All patients (n = 191)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;Low LRR risk&quot; (n = 48)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;High LRR risk&quot; (n = 143)</td>
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<tr>
<td><strong>n (%)</strong></td>
<td><strong>n (%)</strong></td>
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<td><strong>Age (years)</strong></td>
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<tr>
<td>Range</td>
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<tr>
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<td>63 (33–68)</td>
<td>9 (36–68)</td>
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<tr>
<td>≥50 years</td>
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<tr>
<td><strong>Menopausal status</strong></td>
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<td>9 (36)</td>
</tr>
<tr>
<td>Postmenopausal</td>
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<td>16 (64)</td>
</tr>
<tr>
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<tr>
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<td>56 (29)</td>
<td>6 (24)</td>
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</tr>
<tr>
<td>&gt;50 mm</td>
<td>28 (15)</td>
<td>4 (16)</td>
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</tr>
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</tr>
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<tr>
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<td>3 (12)</td>
</tr>
<tr>
<td>Other carcinomas</td>
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<td>1 (4)</td>
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<tr>
<td><strong>ER status</strong></td>
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</tr>
<tr>
<td>Positive</td>
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<td>1–3</td>
<td>98 (51)</td>
<td>15 (60)</td>
</tr>
<tr>
<td>≥4</td>
<td>82 (43)</td>
<td>9 (36)</td>
</tr>
</tbody>
</table>

**a**The Fisher exact test for comparison between "low LRR risk" versus "high LRR risk."

**b**ER status determined by IHC (10% cutoff). Supplemented with status determined from biochemistry measurements, if missing values (29 pts.).

**c**HER2 status was determined by IHC (HercepTest) and FISH if equivocal (2+). Supplemented with status determined from gene expression, if missing values (31 pts.).
genes (Supplementary Document S2). The gene-expression levels of these 4 only 4 of the 7 interaction genes as described in Supplementary Table S4). The DBCG-RT profile was modified according to availability of suitable PCR assays and (Supplementary Table S3) and did not details, see Supplementary Document S2). The 146 patients with small tumors and few involved lymph nodes (T1–2, 1–3 positive lymph nodes; Supplementary Fig. S3D).

The technical transfer of the DBCG-RT profile to qRT-PCR was successfully carried out in 146 of 191 patients (for details, see Supplementary Document S2). The 146 patients were representative for the training set in terms of clinicopathologic variables (Supplementary Table S3) and did not differ statistically significantly from the 45 patients excluded (Supplementary Table S4). The DBCG-RT profile was modified according to availability of suitable PCR assays and success rate of detecting the genes in FFPE. Therefore, the FFPE/qRT-PCR-modified DBCG-RT profile was based on only 4 of the 7 interaction genes as described in Supplementary Document S2. The gene-expression levels of these 4 genes (IGKC, RGS1, ADH1B, and DNALI1) correlated significantly with the expression levels measured by array technology on the corresponding FFPE/qRT-PCR. The FFPE/qRT-PCR-modified DBCG-RT profile provided the same predictive value, when stratified according to PMRT (Fig. 2C and D and Table 2), as the FFT/array-based classification (Fig. 2A and B). The FFPE/qRT-PCR index cutoff value for dividing patients into "high LRR risk" and "low LRR risk" patients, respectively, was –1.1.

Validation of the predictive impact in the DBCG82bc cohort

Detection of the 4 genes in the FFPE/qRT-PCR-modified DBCG-RT profile was successful in 112 validation samples (Supplementary Fig. S2). Using the defined FFPE/qRT-PCR index cutoff value of –1.1 as determined in the technical transfer part of the analysis, 22 patients (20%) were designated as "low LRR risk" and 90 patients (80%) as "high LRR risk." The predictive value was validated in the 112 patients, and a significant benefit from radiotherapy in terms of local control could be seen in the "high LRR risk" patients (30% vs. 7%; P = 0.003; adjusted HR, 0.09; 95% CI, 0.02–0.31; Fig. 2E), whereas "low LRR risk" patients did not show benefit (8% vs. 0%; P = 0.30; adjusted HR not estimated; Fig. 2F; see Table 2 for unadjusted HRs). There was no statistically significant difference in the distribution of clinicopathologic variables between the two risk groups, except for HER2 (P = 0.04; Supplementary Table S1).

When combining tumor size and number of positive lymph nodes into subgroups, there was no statistically significant difference in the distribution of the two risk groups as determined by the DBCG-RT profile for any of the three patient cohorts (Supplementary Table S5).

Discussion

The present study is the first to define and validate a gene profile associated with risk of LRR in patients not receiving PMRT, and predictive of benefit from adjuvant PMRT in a cohort of patients with high-risk breast cancer (DBCG82bc) treated with adjuvant systemic treatment and randomized to receive or not to receive PMRT.

Prognostic gene-expression profiles predicting risk of LRR after breast conserving therapy (BCT; refs. 33–36) and after mastectomy (37) have been published previously, but none of them have been successfully validated. The study by Cheng and colleagues (37) identified 34 genes with significant association with LRR. There is no overlap between the 34 genes and the genes identified in this study. Whether there is any overlap with the genes in a 10-gene signature predictive of cellular radiosensitivity that has been developed from microarray analysis on 35 cell lines (38), and subsequently clinically validated, primarily in terms of risk of DM, in two breast cancer data set (39). Nuyten and colleagues (33) and Kreike and colleagues (40) did not identify a specific geneset predicting risk of recurrence after BCT, though a gene profile based on the wound response signature was described as being of independent prognostic value. Later, the same group developed a 111-gene signature (35), but it did not show independent prognostic value in multivariate analysis, and lost prognostic impact when tested in independent cohorts (34, 41). The gene profile by Nimeus-Malmström and colleagues (34), aiming to
identify patients developing LRR despite radiotherapy after BCT, could not be validated in other cohorts (35, 36, 41), and the most recent study by Sabatier and colleagues (36) did not succeed in determining a robust prognostic signature of LRR.

The two LRR risk groups in the present study could be identified among all clinicopathologic subgroups, and even among patients traditionally considered to have a high risk of LRR (e.g., tumor size >5 cm and >4 positive lymph nodes), the DBCG-RT profile identified patients with a very low risk

| Table 2. HRs for patients treated with or without PMRT in the "low LRR risk" and "high LRR risk" groups; presented unadjusted and adjusted for lymph node status and locally advanced disease, with 95% CIs |
|-----------------------------------------------|---------------|---------------|---------------|
| Training set, FFT | All patients | "Low LRR risk" | "High LRR risk" |
| | HR (95% CI) | HR (95% CI) | HR (95% CI) |
| PMRT, unadjusted | 0.23 (0.12–0.44) | 1.20 (0.17–8.52) | 0.17 (0.09–0.35) |
| PMRT, adjusted | 0.20 (0.10–0.38) | 1.13 (0.14–9.15) | 0.17 (0.08–0.34) |
| Training set, FFPE | All patients | "Low LRR risk" | "High LRR risk" |
| | HR (95% CI) | HR (95% CI) | HR (95% CI) |
| PMRT, unadjusted | 0.26 (0.13–0.53) | 0.61 (0.10–3.72) | 0.22 (0.10–0.48) |
| PMRT, adjusted | 0.23 (0.11–0.48) | 0.58 (0.09–3.83) | 0.20 (0.09–0.45) |
| Validation set, FFPE | All patients | "Low LRR risk" | "High LRR risk" |
| | HR (95% CI) | HR (95% CI) | HR (95% CI) |
| PMRT, unadjusted | 0.13 (0.04–0.46) | n.e. | 0.13 (0.04–0.46) |
| PMRT, adjusted | 0.08 (0.02–0.29) | n.e. | 0.09 (0.02–0.31) |

Abbreviation: n.e., not estimated.
of LRR. Equally, "high LRR risk" patients could be found among patients with 1 to 3 positive lymph nodes or tumor size <2 cm, and benefit from PMRT was observed in these patient groups also. Indication for PMRT could be found even in the subgroup of patients with a combination of small/intermediate tumor size and few involved lymph nodes (T1–2 and 1–3 positive lymph nodes), where indication for PMRT has been controversial and a subject for substantial discussion over the last decade. This indicates that the presented DBCG-RT profile adds information to the conventional parameters used for present-day treatment decision making. The distribution of the risk groups suggests that the ER-negativity adds to the poor prognostic impact of the "high LRR risk" group, contributing to the fact that this study finds a greater benefit from PMRT in ER-negative patients as compared with the preceding study of this cohort (15). In a related study (42), the DBCG-RT profile has been found to be independent of the intrinsic subtypes as determined by either IHC (e.g., ER, PR, and HER2) or gene-expression profiling, and "low LRR risk" and "high LRR risk" groups were found among all subtypes in this study, even among the Luminal A subtype.

Of the seven probes identified from the analysis of FFT, four probes (IGKC, RGS1, ADH1B, and DNALI1) were successfully transferred to corresponding FFPE. One of the probes (hCG2023290) did not recognize a known gene, and a suitable assay could not be found for OR8G2. Of the five genes measured in FFPE, one (HLA-DQA) could only be detected in 10% of the samples and was omitted for further analysis. Explanations to the low success rate of detecting the four genes of the FFPE/qRT-PCR-modified DBCG-RT profile in FFPE samples of the validation set could be, e.g., low yields of mRNA caused by age of the paraffin block or technical issues. The identification of genes in FFPE is dependent on gene-expression level as well as factors related to handling of the paraffin block. The age of the blocks is of great influence, because mRNA degrades with a half-life of 4.6 years (26), implying that the absolute expression values decrease with age of the blocks. This potentially leads to low detection rates further accentuated by low-expressed genes. The initial selection of the genes based on FFT in this study did not take into account that the genes should be highly expressed. The patients were, however, reliably assigned to the two risk groups based on the reduced set of 4 genes.

One of the genes included in the gene profile (IGKC) has previously been reported as prognostic in terms of DM in breast cancer and able to predict response to antracycline-based neoadjuvant chemotherapy (43). IGKC is expected to be derived from mature tumor-infiltrating plasma cells, and as such related to a humoral immune response of presumably protective nature (43). The gene has also been reported as a prognostic marker for DFS and OS in patients with node-negative breast cancer (44). Increased expression of IGKC protein based on IHC in non–small lung cancer has also been associated with improved outcome (45). Elevated expression of this gene might indicate an increased immune response favorable in cancer treatment, and interestingly, among the top correlated genes to several of the seven identified genes, immune response and lymphocyte activation were found to be enriched, when analyzed by the DAVID and Reactome analysis tools. This suggests a potential impact of tumor-infiltrating immune cells on the effect of PMRT on LRR.

RGS1 encodes a regulator of G protein signaling, and the gene has found to be overexpressed in melanoma (46). Furthermore, RGS1 has in combination with two other genes (NCOA3, SPP1) been described as an independent predictor of DFS in malignant melanoma (47) but not in breast cancer. The expression of RGS1 has further been studied in relation to lymphomas and hypoxia (48) and has been described to be associated with HIF1α. The association with hypoxia, known to be a factor related to radioresistance, is consistent with the present findings that tumors with a "low LRR risk" are not showing benefit from PMRT. Both IGKC and RGS1 are included in the FFPE/qRT-PCR-modified DBCG-RT profile. None of the 5 remaining genes have been described in association with intrinsic radiosensitivity, hypoxia, or cell proliferation commonly considered influential in terms of radioresistance.

The increasing incidence of patients with breast cancer and increasing ratio of BCT versus mastectomies subsequently lead to an increasing number of patients who, now and in the future, will be offered adjuvant radiation therapy. The LRR rate has been observed to decrease, and the strategies for administration of radiotherapy must carefully counterbalance the benefits in terms of increased survival and decreased risk of LRR against the risk of secondary morbidity, including ischemic heart disease and secondary cancer. The capability of the presented DBCG-RT profile in identifying patients likely to benefit from PMRT/RT needs to be examined in a prospective study to evaluate the prognostic and predictive impact in patients treated with present-day systemic adjuvant treatment. Whether the prognostic and predictive impact of the DBCG-RT profile is applicable to BCT or not also needs testing in a prospective study.

In conclusion, we have identified and validated a DBCG-RT profile containing prognostic and predictive impact in relation to adjuvant radiotherapy after mastectomy. The gene profile allowed the identification of patients not benefitting from radiotherapy in terms of LRR, and describes characteristics not already embraced by clinicopathologic variables.

**Ethical considerations**: The study of the DBCG82bc cohort has been approved by the Regional Ethical Committee (Journal number 20030263).

**Reporting Recommendation for Tumor Marker Prognostic studies (REMARK) criteria** were adhered to, when reporting the results of the study (49).

**Disclosure of Potential Conflicts of Interest**

T. Tramm, H. Mohammed, S. Myhre, J. Alsner, A.-L. Børresen-Dale, T. Sørlie, A. Frigessi, and J. Overgaard report holding a patent on the presented gene-profile (international patent publication no. WO 2013/132354 A2). No other potential conflicts of interest were disclosed by the other author.
Disclaimer

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References

16. Overgaard M, Nielsen HM, Overgaard J. Is the benefit of postmas- tectomy irradiation limited to patients with four or more positive nodes, as recommended in international consensus reports? A subgroup analysis of the DBCG 82 b&c randomized trials. Radiother Oncol 2007;82:247–53.
Development and Validation of a Gene Profile Predicting Benefit of Postmastectomy Radiotherapy in Patients with High-Risk Breast Cancer: A Study of Gene Expression in the DBCG82bc Cohort

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