

# A Robust Five-Gene RT-PCR Molecular Grade Index Stratifies Recurrence Risk for Grade 2 Tumors, Predicts Chemo-sensitivity and is Compatible with Formalin-Fixed Paraffin-Embedded Tissue



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## BACKGROUND

Historical tumor grade is a well-established prognostic factor for breast cancer that predicts chemotherapy benefit in node-negative breast cancer patients<sup>1,2</sup>. Recently, Page, Allred and coworkers demonstrated that patients with high grade benefited from chemotherapy versus no benefit observed for patients with low and intermediate grade.<sup>3</sup> However, the Breast Task Force in revising the AJCC Cancer Staging Manual decided not to incorporate histologic grade into the TNM staging system due to ambiguity of grade 2's and the lack of information regarding histologic grade as it relates to clinical outcome for small tumors (T1 and T2).<sup>3</sup>

We hypothesized that an objective, quantitative and simple gene expression index for tumor grade (called molecular grade index or MGI) could be developed that is predictive for both clinical outcome and chemo-sensitivity.

## PATIENTS AND TUMOR SAMPLES

**PUBLIC MICROARRAY DATASETS.** Two previously published microarray datasets (accessions GSE3494, GSE1456) were downloaded from Gene Expression Omnibus (GEO, <http://ncbi.nlm.nih.gov/geo>). GSE3494 (Uppsala cohort) consists of 251 patients derived from a population-based cohort treated in Uppsala County, Sweden, from 1987 to 1989, and they were heterogeneous in terms of adjuvant systemic therapy received (untreated or endocrine and/or chemotherapy-treated). Clinical outcome data (breast cancer-specific death) were available for 236 patients with a median follow-up of 10 years. GSE1456 (Stockholm cohort) consists of a similar series of 159 breast cancer patients treated at the Karolinska Hospital, Stockholm, Sweden from 1994 to 1996. Both GSE3494 and GSE1456 contain gene expression data from frozen tumor samples analyzed on the Affymetrix U133A and U133B arrays (Affymetrix, Santa Clara, CA). For determining the performance of the MGI index to predict chemo-sensitivity, MGI was calculated within the previously published raw microarray dataset from Hess, KR, Anderson, K, Symmans, W, et al.<sup>5</sup> The same pre-determined cut-point of "0" was applied to determine predictive performance.

**MGH COHORT.** The 239 pt. MGI cohort used a retrospective case-cohort design<sup>6</sup> and was derived from 683 stage I-III patients with estrogen receptor-positive breast cancer treated at the Massachusetts General Hospital from 1991 to 1999. Clinical follow-up data were obtained from tumor registry and hospital records. Cases were all patients who developed distant metastasis during follow-up; controls were randomly selected from patients who remained disease-free at last follow-up to achieve a 2:1 ratio of controls to cases. In addition, controls were frequency-matched to cases with respect to adjuvant therapy and time of diagnosis. The final cohort consisted of 79 cases and 160 controls, and its patient and tumor characteristics were summarized in Table 1. This study was approved by local Institutional Review Boards.

## REAL-TIME RT-PCR ASSAY for MGI

We designed primer/probe sequences for the five molecular grade genes (BUB1B, CENPA, NEK2, RACGAP1 and RRM2) using Primer Express (ABI). In addition, for the normalization genes, we used primer/probe sequences for ACTB, HMBS, SDHA and UBC described previously.<sup>7</sup> For each FFPE sample, two 7-µm tissue sections were used for RNA extraction; gross macro-dissection was used to enrich for tumor content. RNA extraction, reverse transcription, and TaqMan RT-PCR using the ABI TaqMan instrument (Applied Biosystems, Inc) were performed as described before.<sup>7</sup> The cycling threshold numbers (CTs) were normalized to the mean CT of four reference genes (ACTB, HMBS, SDHA and UBC); normalized CTs were taken to represent relative gene expression levels.

## CALCULATION OF MGI (and GGI)

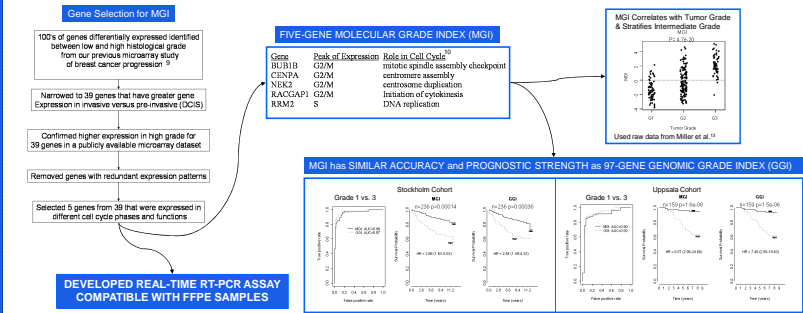
Normalized expression levels for the five molecular grade genes from microarrays or RT-PCR were standardized to a mean of 0 and standard deviations within each dataset and then combined into a single index per sample via principle component analysis (PCA) using the first principle component. Genomic Grade Index (GGI) was calculated from microarray data using the 128 Affymetrix probe sets representing 97 genes and scaled within each dataset to have a mean of -1 for grade 1 tumors and +1 for grade 3 tumors as described before.<sup>7</sup>

## CUT-POINTS & STATISTICAL ANALYSES

**MGI CUT-POINT.** The calculation and the cutpoint for MGI were defined without using any clinical outcome data. Initial analysis of MGI in the Uppsala cohort indicated good discrimination of grade 1 and grade 3 tumors using the mean (0) as cutpoint, and model-based clustering of MGI also indicated a bimodal distribution with a natural cutoff around 0. This cutpoint was further supported by receiver operating characteristic (ROC) analysis described previously.<sup>8</sup>

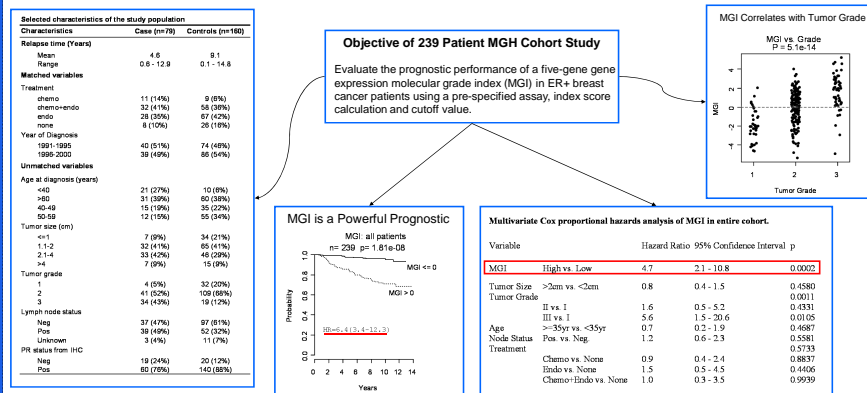
**GENOMIC GRADE INDEX (GGI).** GGI was dichotomized at the cutpoint of 0 as described previously.<sup>8</sup>  
**STATISTICAL ANALYSES.** Kaplan-Meier analysis with logrank test and Cox proportional hazards regression were performed to assess the association of gene expression indexes with clinical outcome. Multivariate Cox regression models were performed to assess the prognostic capacity of gene expression indexes after adjusting for known prognostic factors.

## MOLECULAR GRADE INDEX (MGI) IS A FIVE-GENE INDEX THAT RECAPITULATES TUMOR GRADE 1 & 3 and STRATIFIES INTERMEDIATE GRADE (G2) <sup>6</sup>

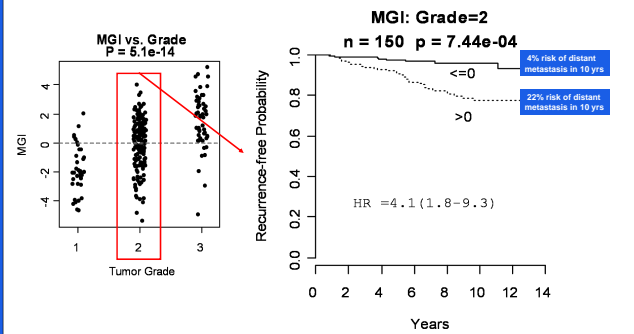


## 239 PATIENT MGH/HARVARD STUDY USING FFPE-COMPATIBLE 5-GENE MGI RT-PCR ASSAY <sup>6</sup>

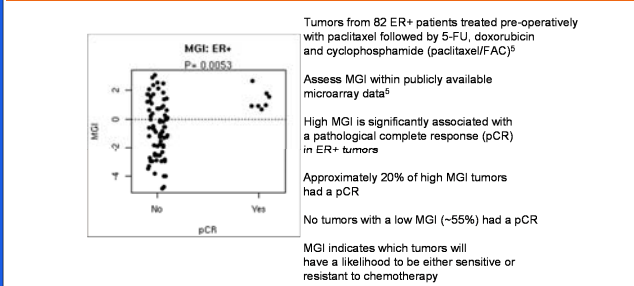
MGI is a POWERFUL PROGNOSTIC INDEPENDENT OF SIZE, GRADE, AGE, NODE STATUS & TREATMENT



## MGI STRATIFIES GRADE 2'S INTO TWO SIGNIFICANTLY DISTINCT OUTCOMES >5-fold difference in risk of distant metastasis in 10yrs <sup>6</sup>



## MGI PREDICTS PATHOLOGICAL COMPLETE RESPONSE TO PACLITAXEL/FAC



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