

Strong Time Dependence of the 76-Gene Prognostic Signature for Node-Negative Breast Cancer Patients in the TRANSBIG Multicenter Independent Validation Series

Christine Desmedt,¹ Fanny Piette,² Sherene Loi,¹ Yixin Wang,⁴ Françoise Lallemand,¹ Benjamin Haibe-Kains,^{1,3} Giuseppe Viale,⁵ Mauro Delorenzi,⁶ Yi Zhang,⁴ Mahasti Saghatchian d'Assignies,⁷ Jonas Bergh,⁸ Rosette Lidereau,⁹ Paul Ellis,¹⁰ Adrian L. Harris,¹¹ Jan G.M. Klijn,¹² John A. Foekens,¹² Fatima Cardoso,¹ Martine J. Piccart,¹ Marc Buyse,² and Christos Sotiriou¹
on behalf of the TRANSBIG Consortium

Abstract Purpose: Recently, a 76-gene prognostic signature able to predict distant metastases in lymph node-negative (N⁻) breast cancer patients was reported. The aims of this study conducted by TRANSBIG were to independently validate these results and to compare the outcome with clinical risk assessment.

Experimental Design: Gene expression profiling of frozen samples from 198 N⁻ systemically untreated patients was done at the Bordet Institute, blinded to clinical data and independent of Veridex. Genomic risk was defined by Veridex, blinded to clinical data. Survival analyses, done by an independent statistician, were done with the genomic risk and adjusted for the clinical risk, defined by Adjuvant! Online.

Results: The actual 5- and 10-year time to distant metastasis were 98% (88-100%) and 94% (83-98%), respectively, for the good profile group and 76% (68-82%) and 73% (65-79%), respectively, for the poor profile group. The actual 5- and 10-year overall survival were 98% (88-100%) and 87% (73-94%), respectively, for the good profile group and 84% (77-89%) and 72% (63-78%), respectively, for the poor profile group. We observed a strong time dependence of this signature, leading to an adjusted hazard ratio of 13.58 (1.85-99.63) and 8.20 (1.10-60.90) at 5 years and 5.11 (1.57-16.67) and 2.55 (1.07-6.10) at 10 years for time to distant metastasis and overall survival, respectively.

Conclusion: This independent validation confirmed the performance of the 76-gene signature and adds to the growing evidence that gene expression signatures are of clinical relevance, especially for identifying patients at high risk of early distant metastases.

In current clinical practice, the majority of patients with early breast cancer receive some form of systemic adjuvant therapy (chemotherapy and/or endocrine therapy), which may have important side effects and which puts considerable burden on health care costs. Although guidelines have been developed

to assist clinicians in selecting patients who should receive adjuvant therapy, it still remains a challenge to distinguish those patients who would really need adjuvant systemic therapy from those who could be spared such treatment. Recognizing the complexity of disease progression, two independent groups

Authors' Affiliations: ¹Institut Jules Bordet, Université Libre de Bruxelles, Brussels, Belgium; ²International Drug Development Institute, and ³Machine Learning Group, Université Libre de Bruxelles, Brussels, Belgium; ⁴Veridex LLC, a Johnson & Johnson Company, San Diego, California; ⁵European Institute of Oncology and University of Milan School of Medicine, Milan, Italy; ⁶National Center of Competence in Research Molecular Oncology, Swiss Institute of Experimental Cancer Research, Epalinges and the Swiss Institute of Bioinformatics, Lausanne, Switzerland; ⁷Institut Gustave Roussy, Villejuif, France; ⁸Karolinska Institute, Stockholm, Sweden; ⁹Centre René Huguenin and Institut National de la Santé et de la Recherche Médicale, Saint-Cloud, France; ¹⁰Guy's Hospital, London, United Kingdom; ¹¹John Radcliffe Hospital, Oxford, United Kingdom; and ¹²Erasmus Medical Center/Daniel den Hoed Cancer Center, Rotterdam, the Netherlands Received 11/22/06; revised 3/2/07; accepted 3/15/07.

Grant support: European Commission Framework Programme VI grant FP6-LSHC-CT-2004-503426, Breast Cancer Research Foundation, Fonds Heuson (S. Loi), and National Center of Competence in Research Molecular Oncology, a research program of the Swiss National Science Foundation (M. Delorenzi). The work of C. Desmedt, B. Haibe-Kains, and C. Sotiriou was supported by a national grant from the Fonds National de la Recherche Scientifique.

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

C. Desmedt and F. Piette are co-first authors. M. Buyse and C. Sotiriou are co-last authors.

All authors have contributed to this work.

These results were presented at a plenary oral presentation at the European Breast Cancer Conference, March 2006, Nice, France.

Conflicts of Interest: Y. Wang and Y. Zhang are employed by Veridex LLC, a Johnson & Johnson company, which is in the business of commercializing diagnostic products.

Requests for reprints: Breast International Group/TRANSBIG Secretariat, Institut Jules Bordet, 121 Boulevard de Waterloo, 1000 Brussels, Belgium. Phone: 32-2-5413526; Fax: 32-2-5413199; E-mail: TRANSBIG@bordet.be.

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doi:10.1158/1078-0432.CCR-06-2765

conducted comprehensive genome-wide assessments of gene expression profiling to identify broadly applicable prognostic markers. In a series of 78 systemically untreated node-negative (N^-) breast cancer patients younger than 55 years of age, and using the Agilent platform, the Netherlands Cancer Institute in Amsterdam and Rosetta identified a 70-gene prognostic signature reported by van't Veer et al. (1). This signature was then validated on a larger set of 295 young N^- and N^+ breast cancer patients from the same institution (2). Thereafter, Erasmus Medical Center and Veridex identified a 76-gene prognostic signature that could be used to predict the development of distant metastases within 5 years in N^- primary breast cancer patients (irrespective of age and tumor size) who did not receive systemic treatment (3). Importantly, and in contrast to van't Veer et al. (1), this study used Affymetrix technology to build a classification algorithm that considered estrogen receptor (ER)-positive patients separately from ER-negative patients, based on the assumption that the mechanisms for disease progression could differ for these two ER-based subgroups of breast cancer patients. This same group recently confirmed their 76-gene signature in a multicenter cohort of 180 N^- breast cancer patients obtained from different institutions (4).

A feature common to both signatures is that when they are compared with the classification results with two conventional consensus, the St. Gallen and NIH guidelines (5, 6), both signatures correctly identified the low-risk patients not needing treatment. This means that the number of patients that would receive unnecessary treatment could be significantly reduced, emphasizing the advantage of these gene signatures over the clinical guidelines.

TRANSBIG, a network for translational research established by the Breast International Group (BIG), recently conducted a validation study of the 70-gene signature and showed reproducible prognostic value in a series of 302 patients from five different centers and across different statistical facilities (7). In the present study, we report the validation results of the 76-gene signature using the same approach and patient series, but an independent laboratory. In addition, given the long

follow-up period (median, 14 years) in this study, we also investigated the prognostic value of this 76-gene signature over time and intriguingly observed a strong time dependence of the prognostic gene signature.

Materials and Methods

Patient samples. This validation study was carried out with frozen archival tumor materials from breast cancer patients previously described by the TRANSBIG consortium (7). Briefly, these patients were younger than the age of 61 years (median age, 47 years) and had node-negative, T_1 - T_2 (≤ 5 cm) tumors. Patients in this series had been diagnosed between 1980 and 1998 (median follow-up, 13.6 years) and had been seen at six centers: Institut Gustave Roussy, Villejuif, France (IGR); Karolinska Institute, Stockholm and Uppsala University Hospital, Uppsala, Sweden (KI); Centre René Huguénin, Saint-Cloud, France (CRH); Guy's Hospital, London, United Kingdom (GH); and John Radcliffe Hospital, Oxford, United Kingdom (JRH). Patients with previous malignancies (except basal cell carcinoma) and bilateral synchronous breast tumors were excluded. The corresponding paraffin-embedded tumor samples of these patients were sent to the Department of Pathology at the European Institute of Oncology, Milan, Italy, where ER status (using immunohistochemistry) and histologic grade (using the Elston and Ellis method) were determined by the same pathologist, blinded to the clinical and genomic data. The clinical centers were also visited by two independent auditors who carried out source data verification of all data in the validation series. The validation protocol was finalized in September 2005, and all institutional ethics committees approved the use of the tumor materials for the purposes described in this study.

Gene expression analysis. Frozen samples were sent from the clinical centers to the Netherlands Cancer Institute, Amsterdam, where RNA had been extracted for the previous TRANSBIG validation study of the 70-gene profile on the Agilent platform (7), except for the samples from Centre René Huguénin, for which RNA was sent directly to Amsterdam. To carry out the present study, RNAs were then shipped to the Jules Bordet Institute to perform the microarray analyses using the Affymetrix U133a GeneChip blinded to clinical data and independent of Veridex. The quality of the RNA obtained from each tumor sample was assessed via the RNA profile generated by the Agilent bioanalyzer. RNA amplification, hybridization, and image scanning were done according

Table 1. Characteristics of patients in the validation series ($n = 198$), according to the clinical and 76-gene signature risk classifications

No. patients	Clinical low risk		Clinical high risk	
	Gene signature low risk ($n = 16$)	Gene signature high risk ($n = 30$)	Gene signature low risk ($n = 39$)	Gene signature high risk ($n = 113$)
Age (y)				
<41	1	3	9	29
41-50	12	21	12	55
51-60	3	6	18	29
Size (cm)				
<1 (T_{1ab})	4	4	0	1
1-2 (T_{1c})	8	13	9	29
2-5 (T_2)	4	13	30	83
Tumor grade				
Good differentiation	11	12	3	4
Intermediate	5	18	16	44
Poor differentiation	0	0	20	63
Unknown	0	0	0	2
ER				
Positive	16	30	25	63
Negative	0	0	14	50

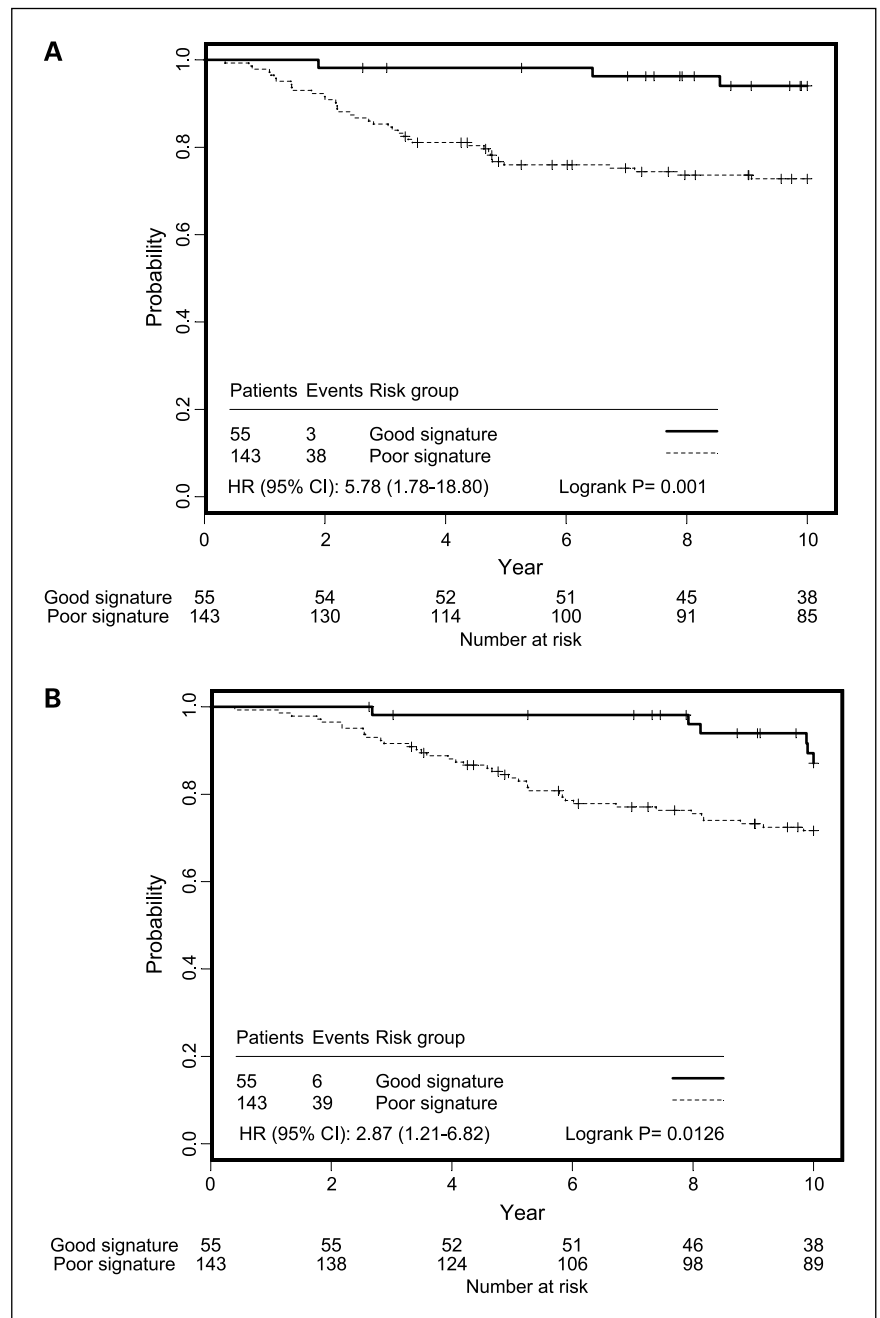


Fig. 1. Kaplan-Meier curves by genomic risk group. The HRs and log-rank tests are stratified by center. *A*, TDM. *B*, overall survival.

to standard Affymetrix protocols. Expression values for each gene were calculated using Affymetrix GeneChip analysis software MAS 5.0. For chip normalization, probe sets were scaled to a target intensity of 600, and scale mask filters were not selected. Chips with signal to noise ratio < 24 were excluded. Each probe set was considered as a separate gene. Genomic high- and low-risk groups were defined by Veridex blinded to clinical data, as described previously (3, 4), using the array based ER assay results and the 76-gene prognostic signature. The raw and normalized gene expression data, together with the patient's characteristics are publicly available on GEO (<http://www.ncbi.nlm.nih.gov/geo>), with accession number GSE 7390.

Statistical analyses. All statistical analyses were carried out by the International Drug Development Institute, Brussels, Belgium, using SAS version 9.1 and SPLUS version 7. Patient clinical data and outcomes were blinded to Jules Bordet Institute and Veridex. The end points considered

in this study were time from diagnosis to distant metastases (TDM), which was the end point used to identify the gene signature (3), and overall survival, defined as time from diagnosis to death from any cause.

The main analytic approach used to validate the gene signature was to estimate hazard ratios (HR), which quantified the relative risk of an event in the high-risk group compared with the low-risk group. HRs were estimated through Cox's proportional hazard regression models, stratified by clinical center to account for the possible heterogeneity in patient selection or other potential confounders among the various centers. HRs for the risk groups defined by the gene signature were estimated with stratification for clinical risk, using the Adjuvant! Online software¹³ to reflect the prognostic effect of the gene signature over and

¹³ <http://www.adjuvantonline.com>

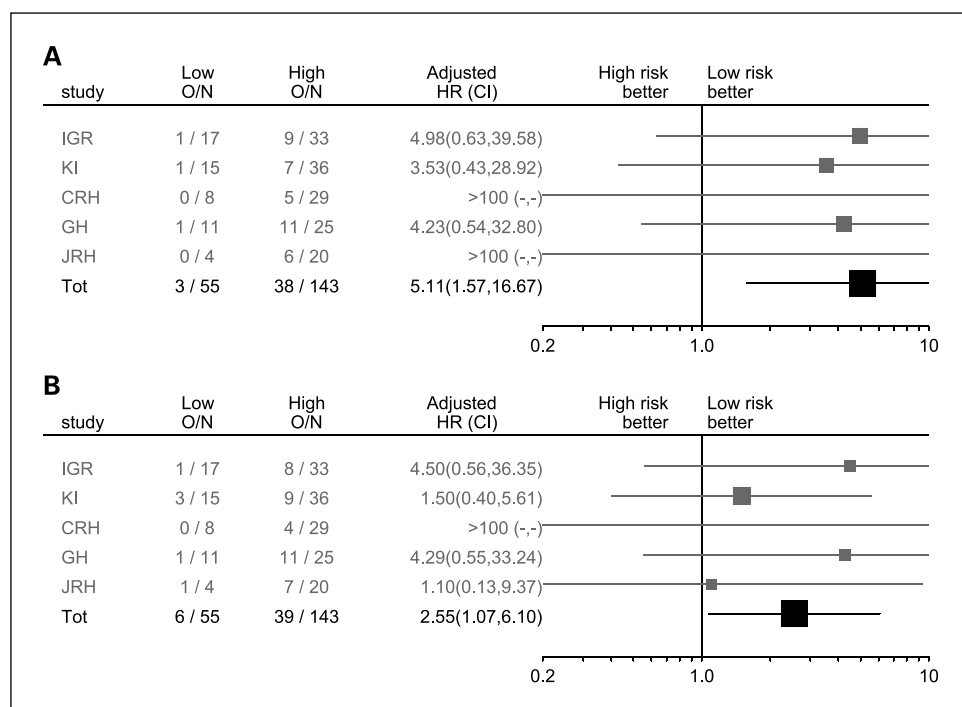


Fig. 2. Forest plots showing HRs of gene signature adjusted for clinical risk [and 95% confidence interval (95% CI)]. HRs are obtained from a Cox model with the gene signature risk category as covariate and the AO risk category as stratification factor. The site was added as a stratification factor for the HR computed on all sites (*Tot*). Low O/N and High O/N, number of observed events/total number of patients in the gene signature low-risk and high-risk groups, respectively. *A*, TDM. *B*, overall survival.

above that of clinicopathologic variables ("adjusted HRs"). This clinical risk classification, which calculates a 10-year survival probability based on a patient's age, tumor size and grade, and ER status (and nodal status, which was negative for all patients considered in the present validation; ref. 8) and had been shown to predict the overall survival, breast cancer-specific survival, and event-free survival in 4,083 women diagnosed with early breast cancer in British Columbia between 1989 and 1993 (9), was also used in the TRANSBIG validation of the Amsterdam 70-gene signature (7). It was decided by consensus of the TRANSBIG consortium members¹⁴ that the low clinical risk group would be defined as patients with a 10-year survival probability of at least 88% if they had >10% of the cells with positive expression of ERs and of at least 92% if they had not (as calculated by Adjuvant! Online). These two cutoffs were chosen to reflect the fact that nowadays, patients with ER-positive tumors receive adjuvant endocrine therapy (with an estimated absolute 10-year survival benefit of about 4% overall), whereas patients in the validation series were all untreated regardless of their receptor status. The adjusted HRs in the different centers were displayed with their 95% confidence intervals on forest plots and tested for heterogeneity. The Kaplan-Meier product limit estimator was used to display time to event curves. "Sensitivity" was defined as the percentage of the patients with distant metastasis within 5 or 10 years that had been predicted correctly by the 76-gene signature. "Specificity" was defined as the percentage of patients free of distant metastasis for at least 5 or 10 years that had been predicted correctly by the 76-gene signature. The effect of the duration of follow-up on HRs was analyzed by censoring all observations at increasing time points. Cox proportional hazard models were fit on the censored data and stratified by center. HRs for the risk groups defined by the gene signature were adjusted for clinical risk by stratifying by the clinical risk groups. HRs for the risk groups defined by Adjuvant! Online were stratified only by center. To be able to compare the results with those of Adjuvant! Online and those of the recently

published validation study of the 76-gene signature (4), the data reported in this study were based on the 10-year follow-up for all the analyses, except for the analysis regarding time dependence.

Results

Patient's characteristics. Out of the 302 patients used for the previous TRANSBIG validation (7), 83 patients were excluded due to insufficient quantities of RNA materials, and an additional 21 patients were excluded because their tumors failed RNA or microarray quality control. Thus, 198 patients were included in the study. No statistically significant difference was found between the original 302 and present 198 patient groups with respect to the patient and tumor characteristics (age, tumor size and grade, ER status, and proportion of patients alive at 10 years). This shows that our population is representative of the overall study population in terms of baseline characteristics. The median follow-up for the 198 patients included was 14.0 years, and distant metastases were found in 51 (26%) of them, with 35 of them showing progression within 5 years (18%). The patients were assessed to high and low genomic risk, as described previously (3, 4), and to high and low clinical risk, as defined by the Adjuvant! Online software¹³ using the pre-defined cutoff. Kaplan-Meier curves for TDM and overall survival as a function of the clinical risk are shown in Supplementary Fig. S1. One hundred forty-three (72%) and 55 patients (28%) were classified as high and low genomic risk, whereas 152 (77%) and 46 (23%) patients were considered to be high and low clinical risk, respectively. The risk criteria were discordant for 69 patients (35%). Interestingly, the low genomic risk group contained 21.9% (14 of 64) of all ER-negative patients, whereas the clinical low-risk group did not contain any. Thus, 25% (14 of 55) of the patients with a low genetic risk were ER negative in contrast to 35% (50 of 143) in

¹⁴ <http://www.breastinternationalgroup.org>

the high genetic risk group. Patient characteristics are shown in Table 1, organized according to genomic and clinical risk.

Effect of the gene signature on TDM and overall survival. The Kaplan-Meier analyses for TDM and overall survival as a function of the gene signature showed statistically significant differences in TDM and overall survival (Fig. 1). Indeed, the actual 5- and 10-year TDM were 98% (88-100%) and 94% (83-98%), respectively, for the good profile group and 76% (68-82%) and 73% (65-79%), respectively, for the poor profile group. The actual 5- and 10-year overall survival were 98% (88-100%) and 87% (73-94%), respectively, for the good profile group and 84% (77-89%) and 72% (63-78%), respectively, for the poor profile group. The HR was 5.78 (95% confidence interval, 1.78-18.80) for TDM and 2.87 (95% confidence interval, 1.21-6.82) for overall survival. The sensitivity for 5- and 10-year TDM was 97% and 93%, respectively, and the specificity was 34% and 31%, respectively.

Gene signature adjusted for the clinical risk. As reported above, the clinical risk was assessed by the Adjuvant! Online

software. We did not consider the St. Gallen criteria and the Nottingham Prognostic Index, as the St. Gallen criteria classify very few patients as low risk, whereas the Nottingham Prognostic Index takes neither the patient's age nor the ER status into account. The adjusted HRs in all centers are shown in Fig. 2. A heterogeneity analysis showed that there were no significant differences between the various centers for both TDM and overall survival. The global HRs remained similar when adjusted for the clinical risk, with a HR of 5.11 (1.57-16.67) for TDM and 2.55 (1.07-6.10) for overall survival. Figure 3 shows the Kaplan-Meier estimates of TDM and overall survival for the four groups of patients as defined in Table 1. Remarkably, in the group of 16 patients defined as both low genomic and low clinical risk, none developed distant metastasis.

Time dependence of the signature. Given the long follow-up period in this study, we investigated the potential of this gene signature to predict distant metastases over time. Interestingly,

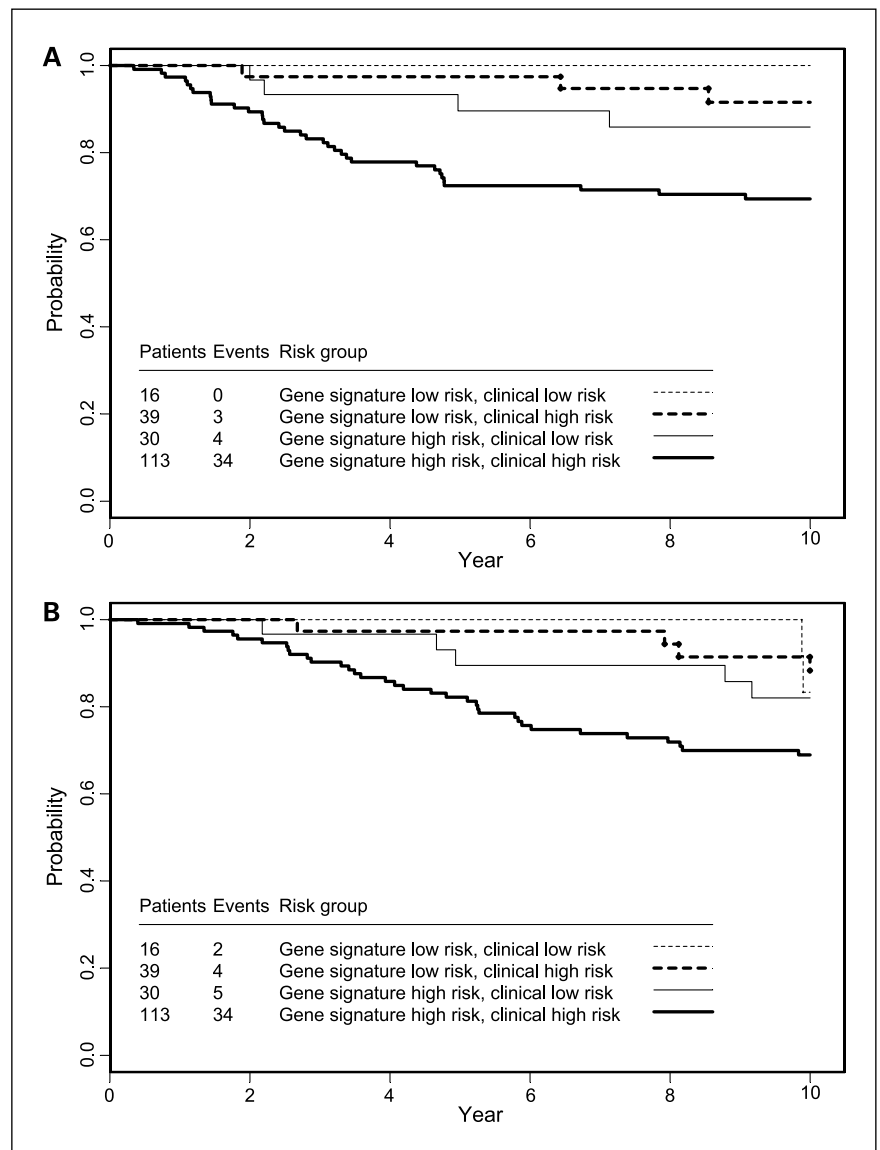


Fig. 3. Kaplan-Meier curves by clinical and genomic risk groups. *A*, TDM. *B*, overall survival.

we observed a strong time dependence of this signature, leading to an adjusted HR of 13.58 (1.85-99.63) and 8.20 (1.10-60.90) at 5 years and 5.11 (1.57-16.67) and 2.55 (1.07-6.10) at 10 years for TDM (Fig. 4) and overall survival (Fig. 5), respectively. In contrast, no such clear time effect was noticed when considering clinical risk on its own, with a HR of 3.29 (1.00-10.87) and 2.11 (0.62-7.21) at 5 years and 2.93 (1.03-8.30) and 1.81 (0.80-4.10) at 10 years for TDM (Fig. 4) and overall survival (Fig. 5), respectively.

Discussion

The Erasmus Medical Center Rotterdam in collaboration with Veridex initially developed a 76-gene signature to identify patients at high risk of distant recurrence (3), which was recently validated by this same group on an independent multicentric population of 180 untreated N⁺ breast cancer patients (4). As it is likely that gene expression profiles will affect future clinical decision making, it has been emphasized that they should be validated independently and preferably by teams outside the original institutions in different series of patients. The results of our study clearly show that the 76-gene

signature remains a powerful prognostic tool even in this particular setting where samples were provided by different institutions, the clinical data audited, the experiments done by an independent laboratory, and the data analyzed by an independent statistical office. Indeed, the proportion of patients remaining distant metastasis-free at 5 and 10 years was similar to the proportion observed in the confirmatory study of Foekens et al. (4), with 98% versus 76% at 5 years and 94% versus 73% at 10 years, for the low- and high-risk groups, respectively. This multicenter independent study correctly predicted 97% of the relapses that occurred within 5 years and 34% of the non-relapsers. These results, which are in agreement with the previous publications (3, 4), provide additional evidence that this gene signature could substantially reduce the number of patients who would be recommended to receive unnecessary adjuvant systemic treatment. Furthermore, it has to be underlined that this gene signature seems to be applicable in different laboratories with the same sensitivity.

The HRs were equal to 5.78 for TDM and 2.87 for overall survival, and these remained similar after adjustment for the clinical risk, as assessed by Adjuvant! Online. This means that the signature adds prognostic information to that provided by

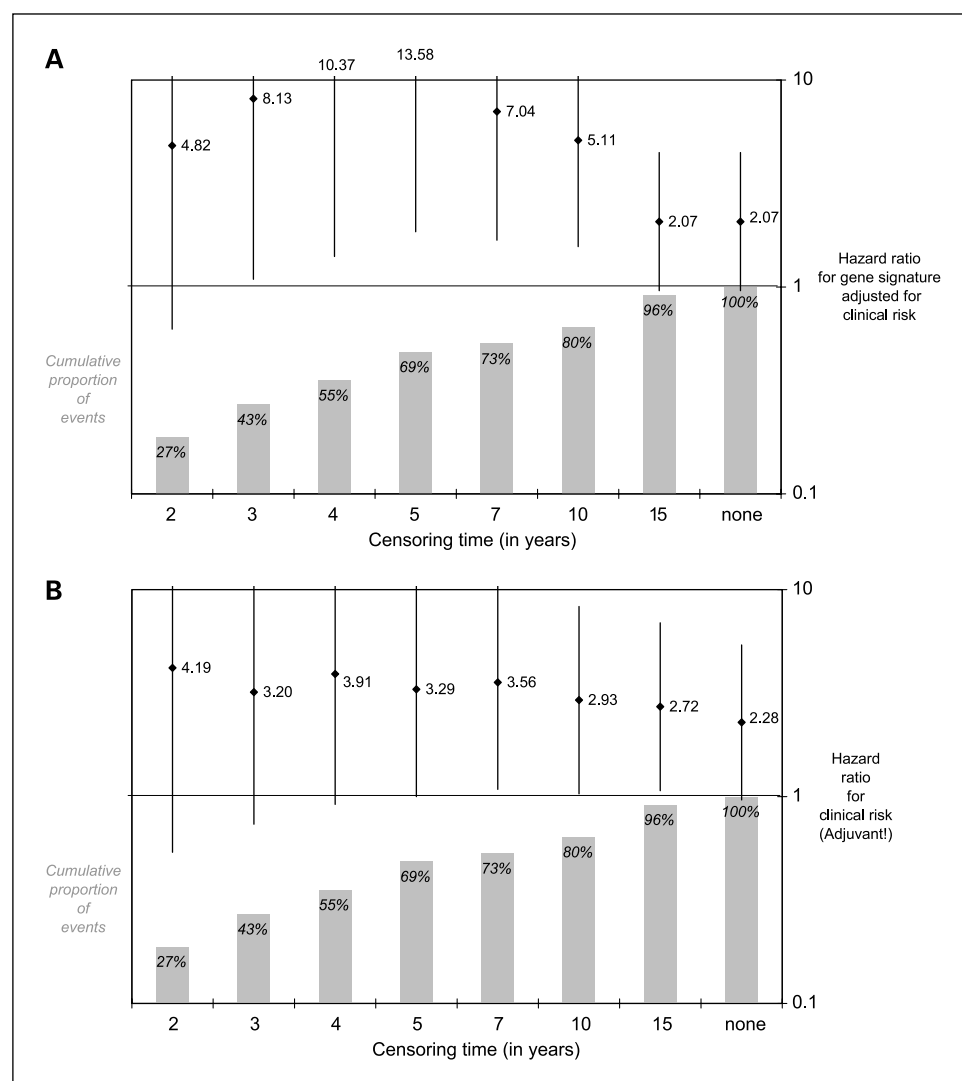
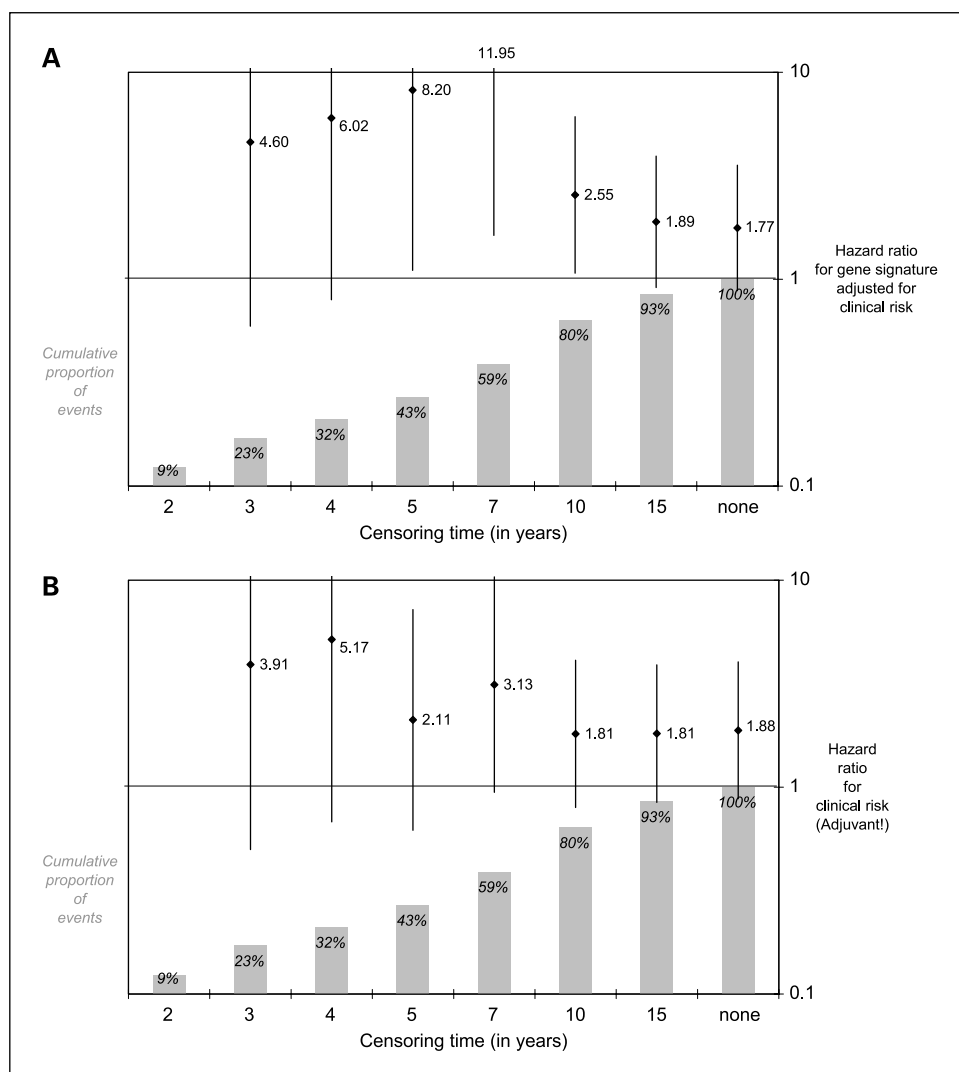


Fig. 4. Time dependence of the gene signature adjusted for the clinical risk (A) and the clinical risk alone (B) for TDM. The HRs are shown together with the 95% confidence interval.

Fig. 5. Time dependence of the gene signature adjusted for the clinical risk (A) and the clinical risk alone (B) for overall survival. The HRs are shown together with the 95% confidence interval.



the clinical risk classification. Furthermore, as reported for the initial population (10), the 76-gene signature was able to identify in this cohort a significant percentage of ER-negative tumors in the low genomic risk group (25%), whereas all ER-negative tumors were considered to be at high clinical risk according to Adjuvant! Online. This is in contrast with the Amsterdam 70-gene signature, which identified only 4% of ER-negative patients (5 of 111) in the low genomic risk group but 44% (85 of 191) in the high genetic risk group (7). The independence of the ER status of the 76-gene signature, together with its independence of age and tumor size, as well as its current validation in an independent series of patients support its strong prognostic power and emphasize its broad clinical applicability.

Given the exceptionally long median follow-up time of this validation cohort (i.e., 14 years), as opposed to a median follow-up of about 8 years for the previously reported populations, we decided to further investigate the behavior of the genomic risk over time. Interestingly, we found that the HR for the gene signature adjusted to the clinical risk was the highest when data were censored at 5 years and decreased as censoring time points increased (11). Similar results were

recently also reported for the validation of the 70-gene signature in the same validation series (7), although the latter included 104 additional patients. This finding highlights that both signatures are a strong predictor of the development of early distant metastases occurring within 5 years, but that their prognostic ability decreases with increasing follow-up years. Nevertheless, the adjusted HR still remained high (5.11) in our study at 10 years of follow-up. These findings, which were not observed for the clinical risk classification, might not be unexpected because the signatures were built to identify patients with distant metastases within 5 years. When these bell-shaped curves of the HR during long-term follow-up will be confirmed in separate series of patients, it does suggest that different mechanisms may be associated with the development of early and late distant metastases. Klein et al. recently provided new insight into the evolution and progression of breast cancer (12, 13). Their results suggested that during breast cancer tumorigenesis some tumor cells may already disseminate in a much earlier genomic state and remain "dormant" until the microenvironment becomes favorable and advantageous aberrations take place in order for distant metastases to develop. However, once metastases start growing, they will still

show the same global genomic phenotype as the primary tumor, as reported by several groups (14–18). Indeed, these dormant cells still have the potential to develop more or less the same genetic aberrations and therefore to show a genetic phenotype similar to what is seen in the primary tumor. Thus, according to Klein's findings and to the current clinical observation that early-stage breast cancer patients still relapse despite complete resection of the primary tumor, we assume that cells may disseminate at different stages of breast cancer tumorigenesis and primary tumor development. Cells that disseminated before primary tumor development would still need to go through some genetic changes and receive the right signals from the local microenvironment before becoming fully active, and this phenomenon could explain the occurrence of late metastases. In contrast, cells that disseminated from the primary tumor in a much more advanced genomic state may already have the genomic background needed for metastatic development and could be responsible for the earlier metastases.

This means that by profiling only primary tumor samples, we might essentially be capturing information regarding tumor cells that disseminated from the primary tumor and not necessarily the young disseminated tumor cells that are in a far less progressed genomic state.

Altogether, these findings may have some important clinical implications. Indeed, in this context, adjuvant therapy might help only to eradicate "active" disseminated breast tumor cells, those which have already acquired the full capacity of unrestrained growth and which are therefore responsible for the development of early metastases. Currently administered adjuvant therapies might not be able to kill the "dormant" disseminated cells, suggesting that new targeted therapies should be developed while late adjuvant chemotherapy or switching long-term endocrine therapy (up to more than 10 years) might be considered.

In conclusion, this independent multicenter validation shows the strong reproducibility and robustness of the 76-gene expression signature and adds to the growing evidence that this classifier is of wide clinical relevance, especially to identify patients at high risk of developing early distant metastases.

Acknowledgments

We thank the many individuals who have made this work possible, including N. Decker and C. Strahle (Breast International Group/TRANSBIG Secretariat, Jules Bordet Institute, Brussels, Belgium) for project coordination.

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Clin Cancer Res 2007;13:3207-3214.

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