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The Molecular Subtype and Tumor Characteristics of Breast Cancer Metastases Significantly Influence Patient Post-Relapse Survival

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Key Message: "An enhanced understanding of the biology of breast cancer metastases is needed to individualize patient management. Here, we show that tumor characteristics of breast cancer metastases significantly influence post-relapse patient survival, highlighting that molecular investigation at relapse offer clinically relevant information, with the potential to improve patient management and survival."

Abstract

Background

We and others have recently shown that tumor characteristics are altered throughout tumor progression, which significantly influences patient survival. These findings emphasize the need for re-examination of tumor characteristics at relapse and have led to recommendations from ESMO and Swedish Breast Cancer group (SweBCG), amongst others. Here we aim to determine whether tumor characteristics and molecular subtypes in breast cancer metastases confer clinically relevant prognostic information for patients.

Patients and Methods:

The translational aspect of the Swedish multicenter randomized trial called TEX included 111 patients with at least one biopsy from a morphologically confirmed loco-regional or distant breast cancer metastasis diagnosed from December 2002 until June 2007. All patients had detailed clinical information, complete follow-up and metastasis gene expression information (Affymetrix array GPL10379).

We assessed the previously published gene expression modules describing biological processes and pathways as well as the intrinsic subtypes (PAM50). Furthermore, by contrasting genes expressed in the metastases in relation to survival, we derived a poor metastasis survival signature.

Results

A significant reduction in post-relapse breast cancer specific survival was demonstrated for patients with the lowest estrogen receptor signaling and apoptosis gene module scores. Similarly, intrinsic subtyping of the metastases provided statistically significant post-relapse survival information (*log-rank P*=0.008), with the worst survival outcome in the basal-like (hazard ratio, 3.7; 95% CI, 1.3 to 10.9) and HER2-enriched (hazard ratio, 4.4; 95% CI, 1.5 to 12.8) subtypes compared with the luminal A subtype. Overall, 25% of the metastases were basal-like, 32% HER2-enriched, 10% luminal A, 28% luminal B, and 5% normal-like. Additionally, the metastases of patients with poor post-relapse survival showed high expression levels of cell-cycle and mesenchymal-related genes.

Conclusions

We show that tumor characteristics and molecular subtypes of breast cancer metastases significantly influence post-relapse patient survival, highlighting that molecular investigations at relapse provide prognostic and clinically relevant information.

/301 words

Key words: Breast cancer metastases, Metastases characteristics, TEX Randomized trial, Gene expression, Gene modules, Biopsy at relapse

This is the translational part of the Swedish multicenter and randomized trial TEX, ClinicalTrials.gov identifier NCT01433614 http://www.clinicaltrials.gov/ct2/show/NCT01433614.

Introduction

Breast cancer is widely recognized as a heterogeneous disease in the sense of both primary tumor metastatic capacity as well as time to metastatic spread of disease. Treatment with endocrine therapy is a major cornerstone in the management of breast cancer and has considerably improved patient survival. However, despite considerable progress, one out of five women with early-stage breast cancer will later develop distant metastatic disease.[1]

We and others have recently shown that tumor characteristics are altered throughout tumor progression,[2–4] which significantly influences patient survival.[2, 5] These findings emphasize the need for re-examination of tumor characteristics at relapse to improve patient management and have led to recommendations from the ABC1, ASCO, ESMO and Swedish Breast Cancer group (SweBCG), amongst others, regarding the reevaluation of metastatic lesions for expression of estrogen (ER), progesterone (PR) and human epidermal receptor 2 (HER2).[6–8]

Here, we aimed to enhance our understanding of the biology of breast cancer metastases in relation to post-relapse survival and to assess tumor characteristics with previously demonstrated prognostic significance in the primary tumor setting to understand their importance in metastatic disease. Our goal in doing so is to provide biologically relevant information that will help to guide individualized patient management. We analyzed tumor characteristics from one or more metastases of 111 breast cancer patients enrolled in the Swedish multicenter prospective and randomized TEX (paclitaxel plus epirubicin plus capecitabine) clinical trial.

Patients and Methods

The Swedish multicenter and randomized trial TEX[9], ClinicalTrials.gov identifier NCT01433614 http://www.clinicaltrials.gov/ct2/show/NCT01433614, enrolled 287 patients with a morphologically confirmed loco-regional or distant breast cancer relapse from December 2002 until June 2007. Previous endocrine treatment for advanced disease in patients with hormone receptor-positive breast cancer was allowed. In addition, previous treatment with an anthracycline, a taxane or 5-FU was allowed if the last course of chemotherapy was given at least 1 year before TEX study entry. Patients were randomized to first-line chemotherapy with a combination of epirubicin and paclitaxel alone or in combination with capecitabine. Detailed clinical information and complete follow-up was available for all included patients.

Patients with metastatic lesions accessible for either a fine-needle aspiration (FNA) or a core biopsy were asked to give a sample, but sampling was optional. We defined the site of relapse as the site where the metastatic biopsy was taken. The most common sites biopsy of metastases were lymph nodes (36.7%), liver (22.5%), skin (18.3%) and breast (15.8%). In total, tumor tissue was available from 149 of 287 patients.

The translational aspect of the trial included 120 relapse biopsies (116 FNA biopsies and 4 core biopsies) from 111 patients yielding sufficient tumor RNA for gene expression profiling. The clinical and pathological patient characteristics of the translational TEX trial were representative of the original TEX trial.

Details of the HRSTA-2.0 custom human Affymetrix array GPL10379 are available at NCBI

GEO depository as GPL10379 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GPL10379). Briefly, this whole genome array contains 52,378 individual probe sets, with each probe set containing 4-20 individual probes. The gene expression microarray data has been deposited into the Gene Expression Omnibus under the accession number of GSE56493.

Approved by the Ethics committee at Karolinska Institutet and the other participating centers. All patients have given written informed consent.

Statistical methods

Preprocessing and Normalization

The relapse tumor gene expression analyses were done in the open source software R using the aroma.affymetrix package.[10] Each gene expression array was individually background corrected and normalized using robust multichip averaging (RMA), no arrays were identified as having poor quality. The quality of the arrays was assessed utilizing Normalized Unscaled Standard Error (NUSE) plots and Relative Log Expression (RLE) plots.

Survival Analyses

Patient follow-up started at the date of the TEX study enrollment and ended at the date of death, or end of study follow-up, July 1, 2013. We performed Kaplan-Meier and multivariate proportional hazard (Cox) analyses adjusting for calendar year and age at diagnosis in addition to the TEX clinical study treatment arms. We did not adjust for additional tumor characteristics due to sample size. The proportional hazard assumption for the main exposure variables was assessed including a time dependent covariate in the survival model. No significant deviation was noted.

Breast cancer specific survival was used as the end point. Post-relapse patient survival was categorized into short-term (up to 1.5 years post-relapse survival) and long-term survival (up to 5 years or more). The short-term survival cut-off (1.5 years post-relapse survival) was defined retrospectively by applying a model of two normal distributions, in order to capture the visually apparent survival distribution, using the mixtools package[11] in R. A full description of this is provided in the Supplementary materials and methods.

Intrinsic subtype (PAM50)

We assessed the intrinsic subtypes (PAM50) for each relapse using the TEX metastases gene expression arrays as previously described, full details are provided in the Supplementary materials and methods.[12]

Gene modules

To characterize the tumor biology of the breast cancer relapses, we applied the set of seven gene expression process modules and ten pathway modules as previously described [13, 14]. Each module is comprised of genes both positively and negatively associated with the gene of interest (e.g. *ESR1*) and the module as a whole is representative of a biological process/pathway. We computed the module score for every gene module in the relapses and divided the resulting continuous variables into tertiles as described in the original publications. The tertiles were grouped so that the most clinically aggressive tertile was compared to the remaining two.

Hierarchical clustering was used to indicate relapse sample similarity with respect to post-relapse survival (short-term or long-term, see categorization above) and relapse site.

Single Probe Association

Differential gene expression according to short-term or long-term post-relapse patient survival (as defined above) was analyzed using the open-source R package OCplus 1.22.0.[15] For additional information see Supplementary Methods.

All data preparation and analysis was done using SAS version 9.3 and R version 2.15.2.

Results

The clinico-pathological characteristics of the 111 patients included in the translational TEX trial are shown in Table 1. The majority of patients were diagnosed with primary breast cancer from 1995 onwards and as expected from a clinically aggressive cohort, there were high numbers of ER negative (37.5%), PR negative (51.5%), grade 3 (52.4%) and stage IV (14.8%) tumors at primary tumor diagnosis. Metastatic sites were distributed as follows: breast (15.8%), liver (22.5%), lung/pleura (1.7%), lymph node (36.7%), skeleton (4.2%), skin (18.3%) and other 0.8%.

Gene expression modules of biological processes reflect the aggressive nature of breast cancer metastases

To characterize the tumor biology of breast cancer metastases, we assessed the set of seven biologically relevant gene expression modules in our 120 metastatic samples.[13] For visualization purposes we selected those genes positively correlated with each module from our samples and performed hierarchical clustering (Figure 1A and 1B). All gene modules (AURKA - proliferation, CASP3 - apoptosis, ERBB2 - HER2 signaling, ESR1 - estrogen signaling, PLAU - tumor invasion/metastasis, STAT1 - immune response and VEGF - angiogenesis) show a varying range of expression values highlighting the biological diversity

between metastases. The aggressive nature of these tumors is also readily apparent with the majority displaying high expression of genes related to proliferation (AURKA module) and approximately half exhibiting low *ESR1* related expression (ESR1 module). The seven patients in our cohort with multiple gene expression arrays (either several biopsies on the same metastatic site or different sites) are indicted with colored arrows and numbers (Figure 1 A, bottom of the heatmap). The paired metastases from four out of seven patients cluster immediately beside one another (numbers 1, 4, 5 and 7). Conversely, three metastatic biopsy pairs from the same patient and relapse cluster separately (numbers 2, 3, and 6), indicating gene expression pattern differences between these intra-relapse tumor biopsies.

Low ESR1 and CASP3 gene module scores are associated with poor post-relapse survival

Long-term and short-term Kaplan-Meier survival curves for the ESR1 and CASP3 gene modules are shown in Figure 2 (Fig. 2 A and B, left panels), and for the remaining modules in Supplemental Figure 1 and 2 (Fig. 1S, 2S). Patients with a low CASP3 module score demonstrated poor long-term survival relative to those with an intermediate/high score (Fig. 2 B, left panel, P=0.0010). In addition, low ESR1 and CASP3 module scores were associated with poor short-term breast cancer specific survival (Fig. 2 A and B, right panels, P=0.0078 and P=0.045, respectively). Furthermore, using a multivariate proportional hazard (Cox) model adjusting for age at diagnosis, diagnosis date and TEX clinical study treatment received, a more than two-fold increased risk for death from breast cancer (short-term survival) was found in patients whose tumors had low CASP3 and ESR1 module scores (Hazard ratio [HR] 2.2; 95% CI, 1.1 to 4.1 and 2.2; 95% CI, 1.2 to 4.2, respectively), see Table 2. No other gene modules were statistically significant (Fig. 1S, 2S and Table 2).

Gene module scores representative of the AKT-MTOR, RAS and BETA-C signaling pathways are associated with poor post-relapse survival

In order to further characterize the biology of our metastatic samples, we extended our gene module analysis to include a set of ten previously described biologically relevant signaling pathways (Ras, MAPK, PTEN, AKT-MTOR, PI3KCA, IGF1, Src, Myc, E2F3 and beta-catenin).[14] In summary, high AKT-MTOR (P-log rank=0.03, HR 1.7; 95% CI, 1.1-2.7), RAS (P-log rank=0.03, HR 1.8, 95% CI, 1.1-2.9), and BETA-C (P-log rank=0.03, HR 1.7, 95% CI, 1.1-2.7) module scores were significantly associated with long-term poor post-relapse survival (Figures 3S-6S).

Breast cancer molecular subtypes of metastases provide information of post-relapse survival

Overall, 25% of the metastases were basal-like (*n*=30), 32% HER2-enriched (*n*=38), 10% luminal A (*n*=12), 28% luminal B (*n*=34), and 5% normal-like. These subtypes were significantly associated with survival in the Kaplan-Meier analysis (*P*= 0.008, Figure 3A) with the shortest survival seen in the basal-like and HER2-enriched subgroups. Furthermore, using a multivariate proportional hazard (Cox) model adjusting for age at diagnosis, diagnosis date and TEX clinical study treatment received, a more than three-fold increased risk for death from breast cancer was found in patients whose tumors were basal-like and HER2-enriched (HR 3.7; 95% CI, 1.3 to 10.9 and 4.4; 95% CI, 1.5 to 12.8, respectively), see Table 2.

Basal-like, cell cycle and mesenchymal-related genes are upregulated in patients with short-term post-relapse survival

Finally, in an effort to understand genetic differences between tumors of patients with short-term vs. long-term survival we performed differential gene expression analysis to identify a poor metastasis survival signature. The poor metastasis survival signature included 136

unique genes differentially expressed between patients with short-term compared to long-term post-relapse survival. The expression of the included genes is presented in Figure 7S and the full gene list is also provided in supplemental Table 1 (Table 1S). Strikingly, patients with short-term survival (Figure 7S, red in the horizontal sidebar) displayed increased expression of the EMT marker *SNAI1*, the cell cycle markers *CCNE1*, *CDC25B* and the basal/triple-negative associated gene *CAV2*. Moreover, the same patients showed reduced expression of the *ESR1*, *GATA3* and *FOXA1* genes, all of which are linked to a luminal breast phenotype and estrogen receptor expression. A gene ontology analysis of these 136 genes is also provided in Supplementary Table S2. These results are in line with our earlier findings showing a worse survival outcome in patients with a low ESR1 module score and basal-like subtype.

Discussion

It is now accepted that standard breast cancer markers alter their expression throughout tumor progression,[2–4, 16] which significantly influences patient survival.[2, 5] As such, investigation of tumor characteristics at relapse has the potential to improve patient management and survival. However, in order to be able to further individualize patient management in the metastatic setting we need a better understanding of the association between metastatic tumor characteristics and patient survival. If tumor aggressiveness as assessed by the prognostic markers in the primary setting can be translated to the metastatic setting, this information would be clinically relevant.

To enhance our current understanding of tumor biology in breast cancer metastases, we analyzed the TEX randomized trial that included 111 patients with available gene expression information from one or more metastatic lesions. Specifically, we assessed gene modules

representative of tumor biological processes and pathways, as well as, the intrinsic subtypes (PAM50)[12] in all metastatic samples and related our findings to patient survival. Interestingly, a significant reduction in post-relapse breast cancer specific survival was demonstrated for patients with the lowest levels of estrogen receptor signaling (ESR1 module) and apoptosis (CASP3). Furthermore, high AKT-MTOR, RAS, as well as BETA-C (beta-catenin) signaling was significantly associated with poor post-relapse survival. Similarly, the PAM50 intrinsic subtypes in the metastases provided statistically significant post-relapse survival information (and displayed higher hazard ratios than gene modules), with the worst survival outcome in the basal-like and HER2-enriched subtypes. Additionally, patients with poor post-relapse survival showed high metastasis expression levels of basal, cell cycle and mesenchymal-related genes with concomitant low expression of luminal genes.

Whilst little is known about the biology of breast cancer metastases themselves, the steps governing the progression from primary breast tumor to seeding of distant metastatic lesions has been a focal point of intense investigation (for review see [17, 18]). These steps comprise what is typically termed "the metastatic cascade" and consist of invasion/proliferation into the tissue surrounding the primary tumour, intravasation to blood or lymph vessels, extravasation to distal organs and finally colonization of the distant tumour microenvironment. With regard to colonization, one of the main processes that has been hypothesised as essential to the successful development of metastatic breast tumours is cell proliferation.[18] Through application of the AURKA gene module we demonstrate that the vast majority of our samples are highly proliferative, this finding is also supported by the PAM50 subtypes where the majority of tumours are luminal B, HER2-enriched and basal-like subtypes – all of which are associated with high levels of proliferation. Of note, the luminal A subtype of tumours which are known to display lower levels of proliferation[19] have the best post-relapse survival in

our dataset. These results may highlight the importance proliferation in relation to survival in metastatic tumours, at least in tumours of luminal subtype, and indicate that a routinely employed proliferative marker such as Ki-67 could also prove informative in a clinical setting.

In conclusion, an enhanced understanding of the biology of breast cancer metastases is needed to improve both patient survival in the metastatic setting and prediction of which patients are at high risk to later develop metastatic breast cancer disease. We show that the tumor characteristics of metastases significantly influence post-relapse patient survival highlighting that molecular investigation at relapse offer clinically relevant information, with the potential to improve patient management and survival in the relapse setting.

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Disclosure

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All remaining authors have declared no conflicts of interest.

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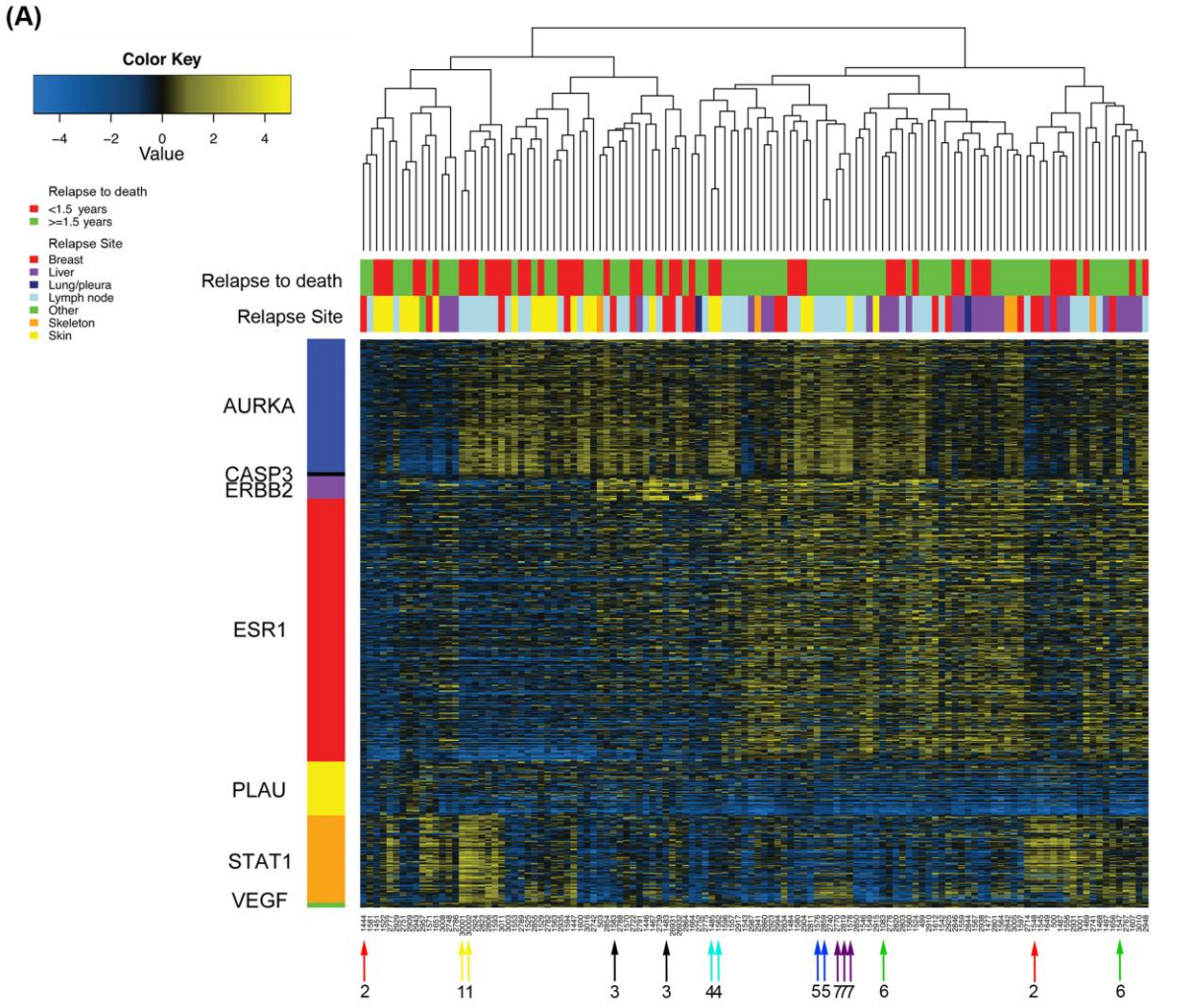
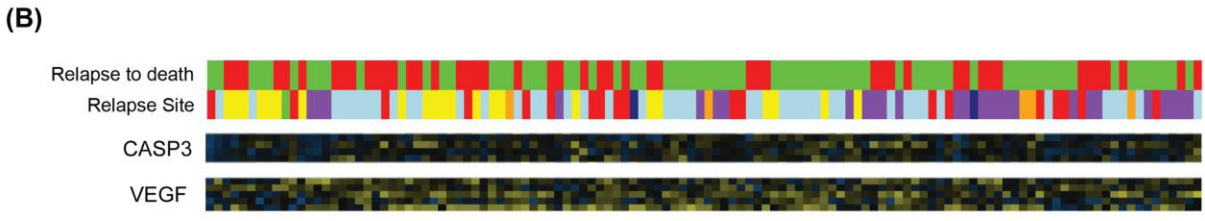
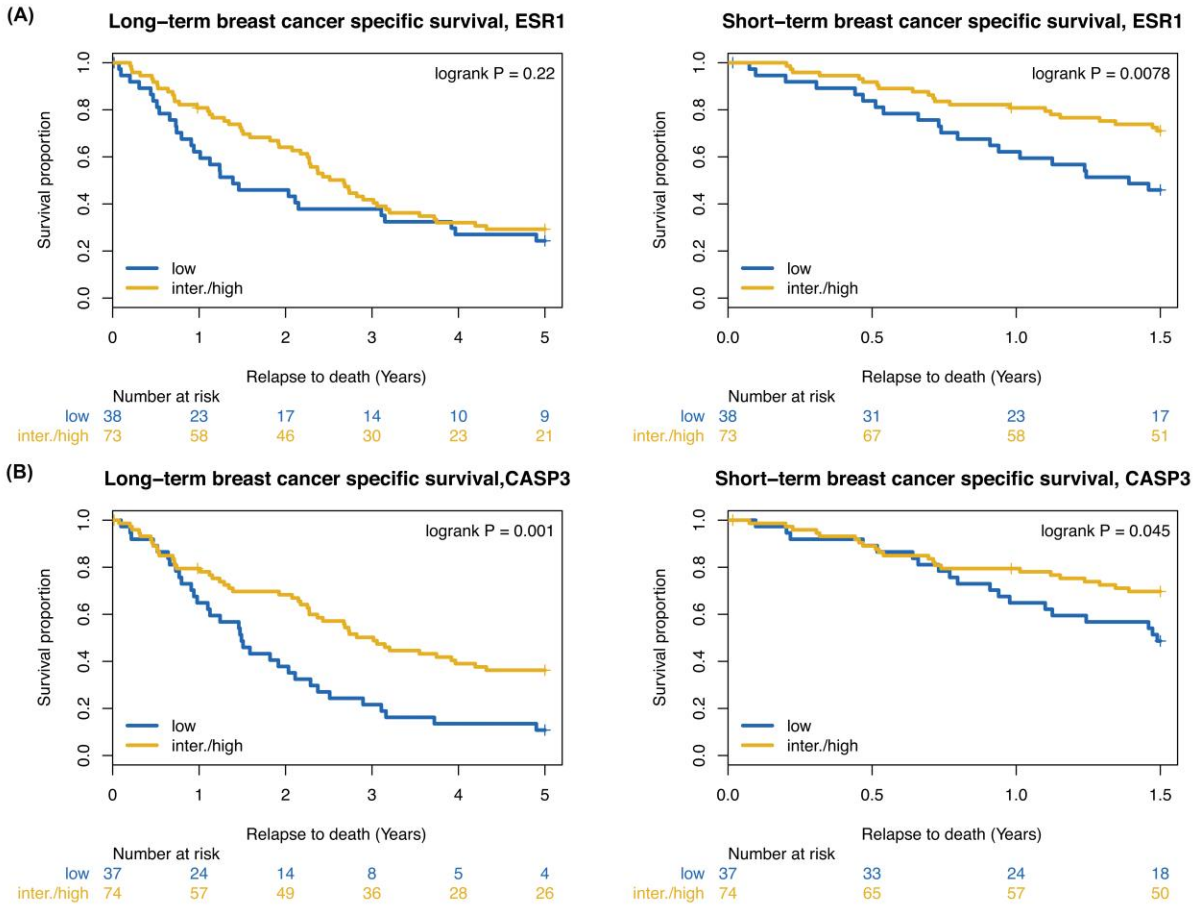
Figure 1. Hierarchical clustering of gene expression profiles of breast cancer metastases based on module genes reflecting seven biological processes (A) Positively correlated module genes were selected for visual representation of 120 breast cancer metastatic samples. Arrows indicate patients with multiple metastases. AURKA= proliferation, CASP3= apoptosis, ERBB2= HER2 signaling, ESR1= estrogen signaling, PLAU= tumor invasion/metastasis, STAT1= immune response, VEGF= angiogenesis. (B) Zoom-in of CASP3 and VEGF modules.

Figure 2. Long-term and short-term breast cancer specific post-relapse survival in relation to gene module groups (A) ESR1 module tertiles long-term (5 years) and short-term (1.5 years) breast cancer specific survival, respectively. (B) CASP3 module tertiles long-term and short-term post-relapse survival, respectively. P-value is based on the log-ranked test, numbers at risk are shown underneath each graph.

Figure 3. The PAM50 intrinsic subtypes in relation to long-term and short-term post-relapse breast cancer specific survival. (A) The PAM50 intrinsic subtypes in relation to long-term (5 years) breast cancer specific survival. (B) The PAM50 intrinsic subtypes in relation to short-term (1.5 years) breast cancer specific survival. P-value is based on log-ranked test, numbers at risk are shown underneath each graph.







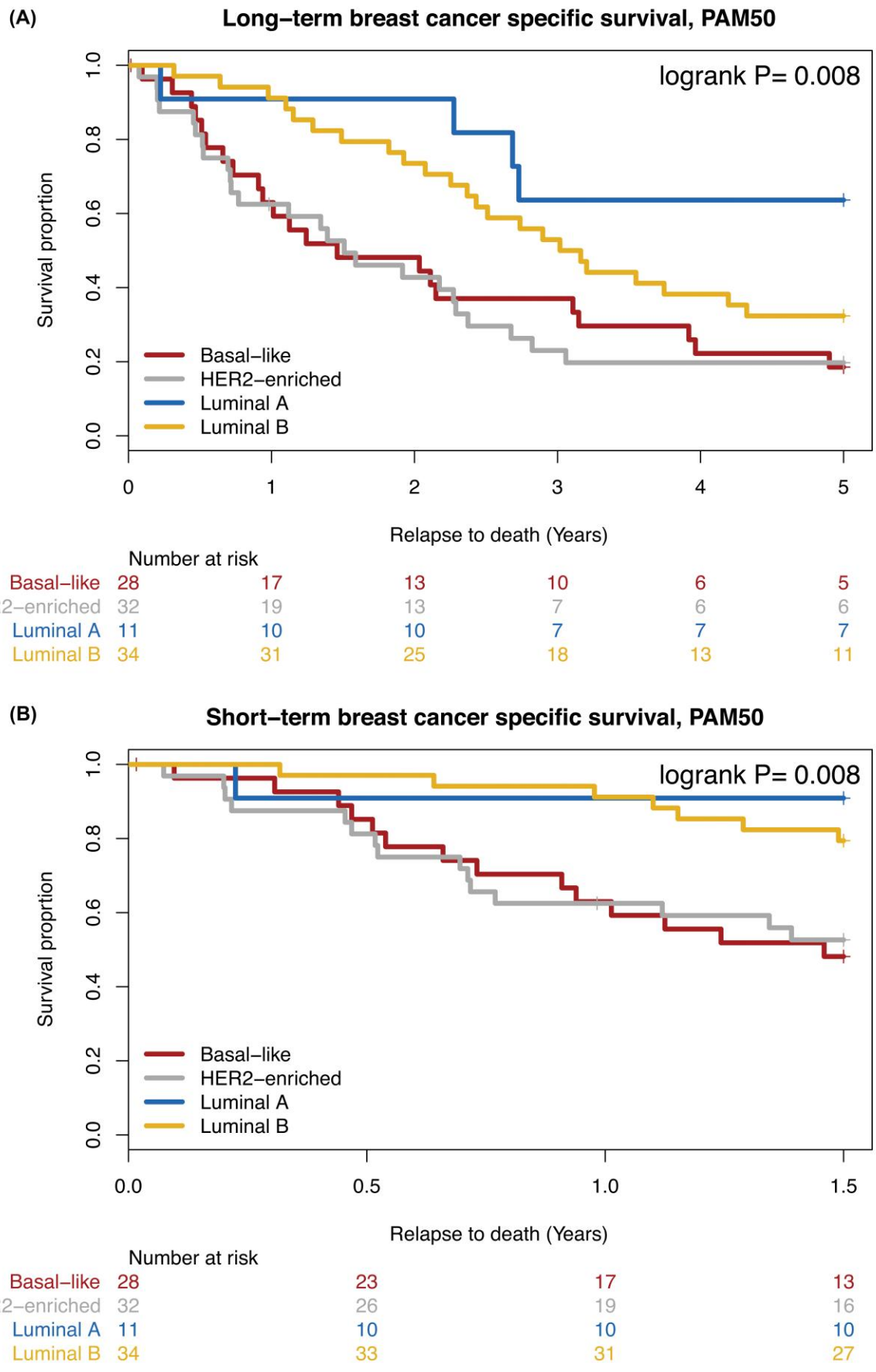


Table 1. Patient and tumor characteristics of the patients included in the translational TEX trial

	Patients	
	Number	Percent
Age at primary tumor diagnosis, years		
<45	27	24.3
45-55	45	40.5
>55	39	35.2
Calendar period of primary tumor diagnosis		
1985-1989	4	3.6
1990-1994	6	5.4
1995-1999	26	23.4
2000-2007	75	67.6
Primary tumor characteristics		
Estrogen receptor status		
Positive	65	62.5
Negative	39	37.5
Unknown	7	-
Progesterone receptor status		
Positive	47	48.5
Negative	50	51.5
Unknown	14	-
Elston-Ellis tumor grade		
1	4	4.9
2	35	42.7
3	43	52.4
Unknown	29	-
T-stage at diagnosis		
T1	36	33.3
T2	43	39.8
T3	13	12.0
T4	16	14.8
Unknown	3	-
N-stage at diagnosis		
N0	32	30.2
N1	65	61.3
N2	8	7.5
N3	1	1.0
Unknown	5	-
M-stage at diagnosis		
M0	85	76.6
M1	26	23.4
Adjuvant therapy		
Endocrine therapy		
Yes	47	42.3
No	64	57.7
Radiotherapy		
Yes	64	57.7
No	47	42.3
Chemotherapy		
Yes	48	43.2
No	63	56.8

Table 2. Multivariate analysis of gene modules and subtypes in relation to patient post-relapse survival

	Long-term breast cancer specific survival			Short-term breast cancer specific survival[#]		
Module[*] (n=111)	HR	95% CI	*P* value	HR	95% CI	*P* value
ESR1 low[§]	1.3	0.8 – 2.1	0.27	**2.2**	**1.2 – 4.2**	**0.01**
ERBB2 high[¥]	0.8	0.5 – 1.4	0.46	0.8	0.4 – 1.5	0.47
AURKA high[¥]	1.2	0.8 – 2.0	0.38	1.1	0.5 – 2.1	0.89
PLAU high[¥]	1.2	0.7 – 1.9	0.52	1.4	0.8 – 2.7	0.25
VEGF low[§]	1.0	0.6 – 1.6	0.95	1.5	0.8 – 2.8	0.21
STAT1 low[§]	1.0	0.6 – 1.6	0.99	0.8	0.4 – 1.6	0.58
CASP3 low[§]	**2.7**	**1.7 – 4.5**	**<0.001**	**2.2**	**1.1 – 4.1**	**0.02**
PAM50[*] (n=105)						
Luminal A (Ref.)	1.0	-	-	1.0	-	-
Luminal B	2.3	0.8 – 6.9	0.12	2.4	0.3 – 19.5	0.42
HER2-enriched	**4.4**	**1.5 – 12.8**	**0.01**	**7.6**	**1.0 – 58.2**	**0.05**
Basal-like	**3.7**	**1.3 – 10.9**	**0.02**	7.2	1.0 – 54.6	0.06

HR= Hazard ratio, CI= Confidence interval
# 1.5 year survival
*Adjusted for age at diagnosis, diagnosis date and treatment received
[§] Intermediate/high as reference group
[¥] Low/intermediate as reference group