Circulating cell-free cancer-testis MAGE-A RNA, BORIS RNA, let-7b and miR-202 in the blood of patients with breast cancer and benign breast diseases

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Background: MAGE-A (melanoma-associated antigen-A) are promising targets for specific immunotherapy and their expression may be induced by the epigenetic factor BORIS.

Methods: To determine their relevance for breast cancer, we quantified the levels of MAGE-A1, -A2, -A3, -A12 and BORIS mRNA, as well as microRNAs let-7b and miR-202 in pre- and postoperative serum of 102 and 34 breast cancer patients, respectively, and in serum of 26 patients with benign breast diseases and 37 healthy women by real-time PCR. The mean follow-up time of the cancer patients was 6.2 years.

Results: The serum levels of MAGE-A and BORIS mRNA, as well as let-7b were significantly higher in patients with invasive carcinomas than in patients with benign breast diseases or healthy women ($P < 0.001$), whereas the levels of miR-202 were elevated in both patient cohorts ($P < 0.001$). In uni- and multivariate analyses, high levels of miR-202 significantly correlated with poor overall survival ($P = 0.0001$). Transfection of breast cancer cells with synthetic microRNAs and their inhibitors showed that let-7b and miR-202 did not affect the protein expression of MAGE-A1.

Conclusions: Based on their cancer-specific increase in breast cancer patients, circulating MAGE-A and BORIS mRNAs may be further explored for early detection of breast cancer and monitoring of MAGE-directed immunotherapies. Moreover, serum miR-202 is associated with prognosis.

Tumour development is usually associated with a change in the global DNA methylation pattern. The DNA methylation process results in the activation of germline-specific genes, such as MAGE-A (melanoma-associated antigen-A), which rely on DNA methylation for repression in somatic tissues (De Smet *et al*, 1999). Identification of antigenic targets that are expressed in breast cancer patients and circulate in blood of high-risk individuals could therefore contribute to the early detection of breast cancer and the development of effective therapeutic approaches and cancer vaccines. Based on their pronounced tumour specificity and their recognition by tumour-specific cytotoxic T cells, cancer-testis antigens (CTAs) that comprise numerous gene families, as well as MAGE-A, are ideal targets for cancer immunotherapy (Caballero and Chen, 2009). The MAGE-A gene family comprises of 12 members (MAGE-A1-12) and is located on chromosome X (De Plaen *et al*, 1994). With exception of testicular germ cells (spermatogonia and primary spermatocytes) and placenta, they are silent in normal somatic tissues, but expressed in numerous epithelial carcinomas and leukemia (Jungbluth *et al*, 2000). However, their individual expression is often heterogeneous and low in tumours.

The Brother of the Regulator of Imprinted Sites (BORIS) is also a cancer-testis gene and involved in the aberrant DNA

demethylation and transcriptional activation (Klenova et al, 2002; Loukinov et al, 2002). This mammalian CTCF paralog was previously described as a transcription factor for epigenetic reprogramming (Loukinov et al, 2002). Alike to MAGE-A, BORIS is expressed in male germ cells and various solid tumour entities resulting in a frequent coexpression of these cancer-testis genes (Klenova et al, 2002). High transcript levels of BORIS have been detected in breast, endometrial, prostate and colon cancer patients. These increased levels of BORIS correlated with the tumour size and grade in these tumour entities (Martin-Kleiner, 2012). Experiments in cell lines have suggested that BORIS expression is sufficient to simultaneously demethylate and activate the transcription of CTAs and oncogenes, such as Myc (Hong et al, 2005; Vatolin et al, 2005; Nguyen et al, 2008; Smith et al, 2009), and may induce the expression of MAGE-A1 (Vatolin et al, 2005). BORIS is considered to be a new oncogene, because it may inhibit apoptosis in cancer cells by activating transcription of hTERT essential for telomerase activity (Renaud et al, 2011).

MicroRNAs (miRs) are a class of naturally occurring small non-coding RNA molecules. Mature miRs consisting of 19–25 nucleotides are single-stranded and derived from hairpin precursor molecules of 70–100 nucleotides. As one of the largest gene families, miRs account for ~1% of the human genome and are highly conserved in nearly all organisms (Kim, 2005). In mammals they are supposed to regulate ~50% of all protein-coding genes (Krol et al, 2010). Mostly, they function as translational repressors, which sequence-specifically bind to complementary sequences in the 3′-untranslational region (UTR) of their target mRNAs. In this way, the protein expression is posttranscriptionally repressed either by inhibiting the translation or degrading the target mRNA (Krol et al, 2010). Cell-free miRs have been identified within blood and their profiles reflect various cancer types suggesting that circulating populations are partly cancer derived (Schwarzenbach et al, 2011). Deregulated levels of let-7b were associated with breast cancer and might, therefore, be potential biomarkers for these patients (Cookson et al, 2012; Schrauder et al, 2012).

In the present study, we evaluated whether the quantification of circulating cell-free MAGE-A1, 2, 3, and 12 and BORIS transcripts in blood could be useful for early detection of breast cancer and be of prognostic relevance based on their relative high expression shown earlier (Wischnewski et al, 2006). As sequence analyses suggest a potential interaction of these cancer-testis genes with let-7b and miR-202, we included these miRs in our diagnostic blood biomarker panel.

MATERIALS AND METHODS

Ethics statement. Serum breast cancer samples were obtained from the Department of Gynecology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany. Written Informed consent for the use of patient data and serum samples had been obtained from all patients in accordance with the local ethics committee according to the principles of the declaration of Helsinki.

Study populations. Serum samples of 102 female patients with primary breast cancer (mean age: 60, range 30–82) undergoing surgery and 26 patients with benign breast diseases (mean age: 59, range 30–82), who received treatment from April 1999 to February 2006 were included in this study. Clinical and histopathological factors (age, nodal status, hormone receptor status, grading, tumour stage and histological subtype) were obtained by reviewing medical charts and pathologic reports. Detailed patient characteristics are listed in Table 1. Patients were treated with surgery, radiotherapy and systemic treatment according to national guidelines. Most patients received adjuvant endocrine treatment and/or

Table 1. Clinicopathological parameters of the breast cancer patients	
Parameters	Patients, n (%)
Total	102 (100)
Age	30–82, median 60
Histology	
Ductal	64 (63)
Lobular	21 (21)
Others	16 (16)
Lymph node	
Negative	54 (53)
Positive	48 (47)
Tumour stage	
pT1-2	45 (44)
pT3-4	57 (56)
Tumour size (cm)	
<2	47 (47)
>2	53 (53)
Grading	
1, 2	57 (56)
3	44 (44)
Oestrogen receptor	
Negative	20 (20)
Positive	80 (80)
Progesterone receptor	
Negative	36 (36)
Positive	64 (64)
Bone marrow status	
DTC negative	66 (65)
DTC positive	36 (35)
Recurrence	
No	32 (40)
Yes	49 (60)
Death	
No	67 (67)
Yes	33 (33)
Chemotherapy	
No	34 (33)
Yes	68 (67)
Endocrine therapy	
No	24 (23)
Yes	78 (77)

chemotherapy. Neither radiotherapy nor neoadjuvant chemotherapy had been performed prior to surgery. The preoperative blood samples of the breast cancer patients were obtained within 2 days before surgery, and the postoperative samples were obtained 2–3 months after surgery. The mean follow-up time of the breast cancer patient group was 6.2 years (range 0.6–11.9 years). Of the 102 breast cancer patients, 36 patients underwent mastectomy. During surgery in the 102 patients, disseminated tumour cells were examined from bone marrow aspirates taken as previously

described (Riethdorf and Pantel, 2008). Of the 26 patients with benign breast tumour (mean age: 53, range 26–97), 46%, 35%, and 19% had fibroadenoma, mastopathy, or other benign breast tumours, respectively. In addition, peripheral blood samples from 37 healthy women (mean age: 62, range 50–85) with no history of cancer and in good health based on self-report were enrolled.

Cell lines. The cell lines BT20, BT474, MDA-MB-468, T47D, MCF-7, GI101 and MDA-MB-231 (breast adenocarcinoma cells) and MTSV1.7 (breast epithelial cells) were cultured in DMEM (Invitrogen, Karlsruhe, Germany) supplemented with 10% FCS (fetal calf serum; PAA Laboratories, Cölbe, Germany), 2 mM L-glutamin (Invitrogen) and under standard conditions (37 °C, 10% CO_2, humidified atmosphere). The micrometastatic cell line BCM1 was cultured at 37 °C, 5% CO_2 and 10% O_2 in RPMI (Invitrogen, Karlsruhe, Germany) supplemented with 10% FCS (PAA Laboratories), 2 mM L-glutamin (Invitrogen), 10 mg ml^{-1} Insulin-Transferrin-Selenium-A (Invitrogen), 50 ng ml^{-1} recombinant human epidermal growth factor, and 10 ng ml^{-1} human basic fibroblast growth factor (both from Macs Miltenyi Biotec, Bergisch-Gladbach, Germany). Cell viability was determined by trypan blue staining.

Extraction of total RNA. Total RNA was extracted from cell lysates using the RNeasy RNA Isolation kit (Qiagen, Hilden, Germany) according to the manufacturer's description. For isolation of total RNA from serum samples, the mirVana PARIS kit (Ambion, Darmstadt, Germany) was used. Four hundred microlitres of serum samples was incubated with an equal volume of denaturation solution for 5 min on ice. RNA extraction was performed with acid-phenol:chloroform followed by ethanol precipitation with a filter cartridge. The extracted RNA was eluted in 100 µl of preheated elution solution and measured on a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The RNA samples were immediately stored at −80 °C.

Conversion of total RNA into cDNA. Reverse transcription for MAGE-A and BORIS was performed by the cDNA Synthesis kit (Thermo Fisher, St. Leon-Rot, Germany). The 20 µl-reverse transcription reaction contained 4 µl 5× Reverse Transcription Buffer, 2 µl 10 mM dNTPs, 1 µl RiboLock Ribonuclease inhibitor, 1 µl oligo(dT)$_{18}$, 2 µl M-MuLV Reverse Transcriptase (20 U µl^{-1}), 5 µl RNA and nuclease-free water. The reaction was performed on a MJ Research PTC-200 Peltier Thermal Cycler (Global Medical Instrumentation, Ramsey, MN, USA) at 37 °C for 60 min and 70 °C for 10 min.

Reverse transcription for let-7b and miR-202 was performed by the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Darmstadt, Germany). The 10 µl-reverse transcription reaction contained 1 µl 10× Reverse Transcription Buffer, 0.1 µl 100 mM dNTPs, 0.13 µl RNase Inhibitor (20 U µl^{-1}), 0.66 µl MultiScribe Reverse Transcriptase (50 U µl^{-1}), 4 µl RNA derived from human serum and 2 µl specific primers. The reaction was performed on a MJ Research PTC-200 Peltier Thermal Cycler (Global Medical Instrumentation) at 16 °C for 30 min, 42 °C for 30 min, and 85 °C for 5 min.

Quantitative real-time PCR of MAGE-A RNA, BORIS RNA and microRNAs in serum samples. For the amplification of MAGE-A and BORIS RNA, 2 µl cDNA of MAGE-A1, -A2 or BORIS as well as preamplified cDNA of MAGE-A3 or -A12 were mixed with 5 µl SYBR Green (Thermo Fisher) and 0.25 µl primer pairs (Sigma-Aldrich, Taufkirchen, Germany) in a 10-µl reaction on a twin-tec real-time PCR plate (Eppendorf, Hamburg, Germany). The following primer pairs were used for MAGE-A1: 5′-GTTCCCGCC AGGAAACATC-3′ and 5′-GAACTCTACGCCGTCCCTCAG-3′, MAGE-A2: 5′-AAGTAGGACCCGAGGCACTG-3′ and 5′-GAAG AGGAAGAAGCGGTCTG-3′, MAGE-A3: 5′-TGGCCGATGTGT

CTATTGAA-3′ and 5′-ACCTTTGCCCAAGTCATCTG-3′, MA GE-A12: 5′-TGGCCGATGTGTCTATTGAA-3′ and 5′-ACCTTTG CCCAAGTCATCTG-3′, BORIS: 5′-CTCAGGTGAGAAGCCTT ACG-3′ and 5′-TGATGGTGGCACAATGGG-3′ and β-actin (reference): 5′-CCTCCCTGGAGAAGAGCTACG-3′ and 5′-AGG ACTCCATGCCCAGGAAG-3′. The quantitative real-time PCR reaction was carried out at 95 °C for 15 min and in 45 cycles at 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s on a Mastercycler Realplex (Eppendorf). Melting curve analyses were performed to verify the specificity and identity of PCR products. The preamplification of MAGE-A3 and -A12 was carried out using 2 µl cDNA, 5 µl Taq PCR Master Mix (Qiagen) and 0.25 µl primer pairs (Sigma-Aldrich) on a MJ Research PTC-200 Peltier Thermal Cycler (Global Medical Instrumentation).

For the miR amplification, miR-specific TaqMan microRNA assays (Life Technologies, Darmstadt, Germany) for let-7b, miR-202 and miR-16 (reference miR) were used. In a 10 µl reaction, 2 µl cDNA were mixed with 5 µl TaqMan Gene Expression Master Mix and 0.5 µl miR-specific TaqMan MicroRNA Assay Mix (Life Technologies). The quantitative real-time PCR reaction was performed at 95 °C for 10 min and in 40 cycles at 95 °C for 15 s and 60 °C for 60 s on a Mastercycler Realplex (Eppendorf).

Transient transfection of let-7b and miR-202. To analyse whether let-7b and miR-202 influence the expression of MAGE-A1, 3×10^5 of MCF-7 and BCM1 cells were seeded in 6-well plates (NUNC, Roskilde, Denmark) and transfected with the double-stranded miScript miRNA mimics hsa-let-7b and hsa-miR-202 or scrambled (negative) control at final concentrations of 10 nM (Qiagen) or with the single-stranded miScript inhibitors hsa-let-7b and hsa-miR-202 at final concentrations of 50 nM (Qiagen) with 2 µl X-tremeGENE HP DNA Transfection Reagent (Roche Diagnostics, Mannheim, Germany). After incubation of 48 h, total RNA and protein were extracted using peqGOLD TriFast (Peqlab, Erlangen, Germany) according to the manufacturer's instructions.

Quantitative real-time PCR and western blot of MAGE-A1 expression in MCF-7 cells. To determine the mRNA expression of MAGE-A1, a total of 200 ng RNA from basal and transfected MCF-7 and BCM1 cells was reverse transcribed using the First strand cDNA synthesis kit (Fermentas, St. Leon-ROT, Germany). The mRNA expression levels were subsequently quantified by real-time PCR using the Maxima SYBR Green/ROX qPCR Master Mix (Fermentas) and the following primers for MAGE-A1: 5′-GTTCC CGCCAGGAAACATC-3′ and 5′-GAACTCTACGCCGTCCCT CAG-3′.

Protein levels of MAGE-A1 in basal and transfected MCF-7 cells were investigated by western blot analysis. Approximately, 30 µg of cell lysates were electrophoretically separated and blotted onto a PVDF membrane (Millipore, Billerica, MA, USA), which was subsequently incubated with antibodies specific for MAGE-A1 (Abcam, Cambridge, UK). The membrane was reprobed with the anti-Hsc70 antibody (Santa Cruz, Heidelberg, Germany) overnight that served as loading control. Detection of the proteins was carried out using peroxidase-conjugated secondary antibodies (Dako, Glostrup, Denmark) and the chemiluminescence ECL detection Kit (Amersham Biosciences, Zürich, Switzerland).

Statistical analyses. The statistical analyses were performed using the SPSS software package, version 18.0 (SPSS Inc. Chicago, IL, USA) and MatLab R2013b (The MathWorks Inc., Ismaning, Germany). Relative expression data were log2 transformed in order to obtain normally distributed data. Statistical difference of mRNA and miR expressions between healthy controls, benign tumour and malignant breast cancer patients were calculated using ANOVA with Tukey's HSD test for all pairwise comparisons that correct for experiment-wise error rate. Two-sample comparisons were performed using Student's t-test for equal or unequal variance

where appropriate, and the Holm–Bonferroni method was employed for multiple comparison correction. Predictive value of expression data was performed using leave-one-out cross-validation (LOOCV) in multinomial logistic regression. Univariate and multivariate analyses were performed for prognostic factors of recurrence-free and overall survival using the Cox regression model. Kaplan–Meier plots were drawn to estimate overall and disease-free survival, and the log-rank test was applied for statistical analyses. Missing data were handled by pairwise deletion. A P-value $\leqslant 0.05$ was considered as statistically significant. All P-values are two-sided.

RESULTS

Heterogeneous expression patterns of BORIS, MAGE-A1, -A2, -A3 and -A12, let-7b and miR-202 in breast cancer cell lines. We first analysed the mRNA expression of BORIS and MAGE-A genes in breast adenocarcinoma cells, breast epithelial cells, and the micrometastatic cells BCM1 (breast cancer). The micrometastatic cells were derived from disseminated tumour cells present in the bone marrow of a breast cancer patient without overt distant metastases (Pantel *et al*, 1995). Interestingly, these cells co-express keratin and vimentin consistent with an EMT (epithelial-mesenchymal transition)-like invasive phenotype as well as other cancer stem cell characteristics (Willipinski-Stapelfeldt *et al*, 2005; Bartkowiak *et al*, 2009; Joosse *et al*, 2012). As shown in Table 2, the basal transcript levels of MAGE-A, BORIS, let-7b and miR-202 were heterogeneous in the investigated cell lines. Only in the breast adenocarcinoma cells MDA-MB-468 and T47D, all four MAGE-A genes and BORIS gene were co-expressed. High expression levels of BORIS could be observed in BCM1 cells, whereas MAGE-A was not expressed in these cells. The ostensible absence of BORIS transcripts in some cancer cells may be due to the used primer pair, which does not amplify all splice variants of BORIS (Renaud *et al*, 2007). Moreover, a negative association between the MAGE-A transcript and both miR levels could not be observed in the cell lines analysed. Whereas let-7b was expressed in each cell line, low levels of miR-202 could only be detected in MDA-MB-231 cells.

Diagnostic relevance of cell-free MAGE-A and BORIS RNA concentrations. Cell-free RNA extracted from blood serum of 102 breast cancer patients, as well as from 26 patients with benign breast disease and 37 healthy women was measured by quantitative real-time PCR. As shown in the box plot of Figure 1, the serum levels of MAGE-A1 ($P<0.001$), MAGE-A2 ($P<0.001$), MAGE-A3 ($P<0.001$) and BORIS RNA ($P<0.001$) were significantly higher in breast cancer patients than in healthy women. Although the

levels of MAGE-A1 ($P=0.043$), MAGE-A2 ($P=0.020$) and BORIS ($P<0.001$) were also elevated in patients with benign disease compared with healthy women, there was a further significant increase in breast cancer patients. Thus, the serum levels of MAGE-A1 ($P<0.001$), MAGE-A2 ($P<0.001$), MAGE-A3 ($P=0.003$) and BORIS RNA ($P=0.020$) could significantly differentiate between benign and malignant breast tumours. In contrast, the serum levels of MAGE-A12 RNA were similar in the three cohorts (Figure 1).

Diagnostic relevance of circulating let-7b and miR-202 concentrations. In addition to the quantification of cell-free RNA, we also determined the levels of the circulating microRNAs let-7b and miR-202. As shown in the box plot of Figure 1, the serum levels of both miRs were significantly higher in breast cancer patients than in healthy women ($P<0.001$). Based on the broad range of circulating miR-202 in the serum of breast cancer patients and in particular of patients with benign disease, only the level of let-7b could significantly differentiate between benign and malignant breast tumours ($P<0.001$, Figure 1).

Classification model to predict tumour status. In order to determine the ability of cell-free RNA and miRs in distinguishing breast cancer patients from patients with benign breast diseases and healthy women, we performed multinomial logistic regression. No interaction was found between the factors. MAGE-A1, BORIS, and miR-202 alone provided the best model (log-likelihood = − 61.468). Using leave-one-out cross-validation, correct classification was made in 22 out of 27 healthy controls, 7 out of 19 benign, and 65 out of 68 malignant cases (Table 3, Supplementary data 1); which is in total an accuracy of 82%. These results indicate that our classification model can reliably predict an individual's disease status ($P<0.00001$, Chi^2 test).

Pre- and postoperative serum levels of circulating RNA and miRs. From 34 breast cancer patients additional serum samples were collected ∼2–3 months after surgery. To obtain information on the impact of surgery on the levels of circulating RNA and miRs, we compared the concentrations before and after surgery. Half of the patients had lymph node metastases. Tumour recurrence was observed in 11 of the 34 patients after a mean time period of 42 months. Fifteen patients underwent a mastectomy. Thirty-one women received chemotherapy and 26 women endocrine treatment.

Although the serum levels of MAGE-A2 and MAGE-A3 RNA decreased postoperatively, the decrease was not significant. However, significant lower levels of MAGE-A1 and BORIS RNA could be observed ($P<0.001$, Figure 2) after surgery. In contrast, the serum levels of miR-202 were significantly higher in breast

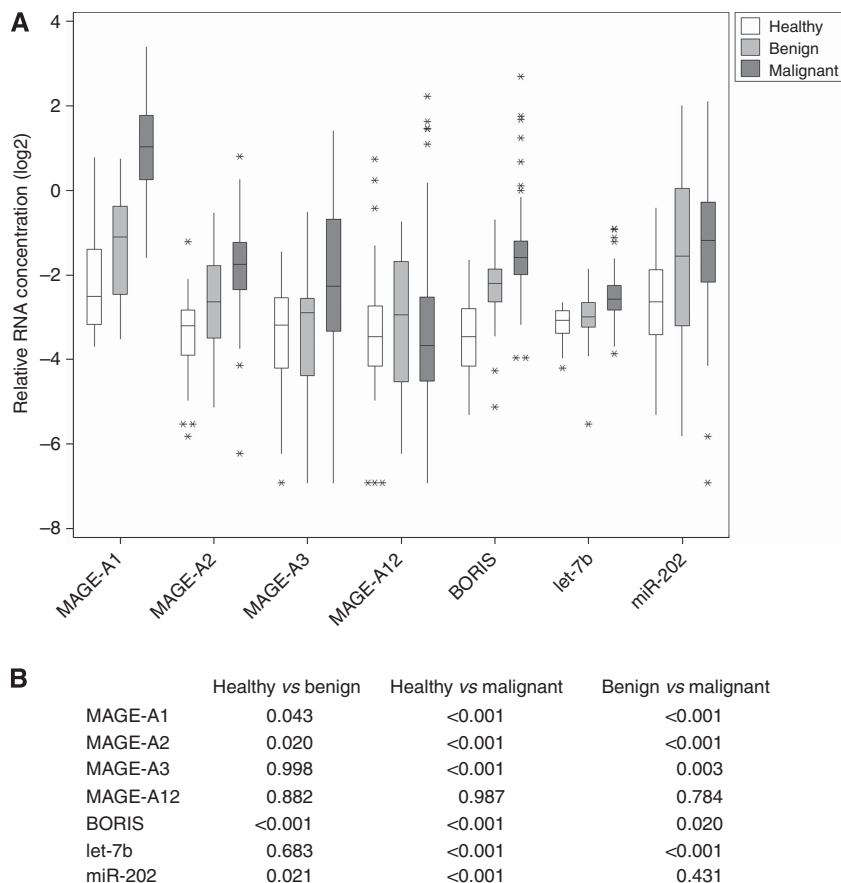| Table 2. MAGE-A, BORIS and miR gene expression in breast cancer cell lines							
Cell lines	MAGE-A1	MAGE-A2	MAGE-A3	MAGE-A12	BORIS	Let-7b	miR-202
BT20	+ +	+ +	+ +	+ +	−	+	−
BT474	+ +	+ +	+ +	+ +	−	+	−
MDA-MB-468	+ +	+ +	+ +	+ +	+ +	+ +	−
T47D	+ +	+ +	+ +	+ +	+ +	+ +	−
MCF-7	+	−	−	−	−	+	−
GI101	−	−	−	−	+ +	+ +	−
MDA-MB-231	−	+	+	−	−	+ +	+
MTSV1.7	+	+	+	−	+	+	−
BCM1	−	−	−	−	+ +	+	−
No −, weak +, strong + + mRNA expression.							

Figure 1. Circulating MAGE-A1, -A2, -A3, -A12 and BORIS RNA, and let-7b and miR-202 levels in breast cancer patients, patients with benign breast disease and healthy women. Box plots showing the different amounts of MAGE-A1, -A2, -A3, -A12 and BORIS RNA, and let-7b and miR-202 that circulate in the blood of healthy individuals ($n = 37$), patients with benign breast disease ($n = 26$) and breast cancer patients ($n = 102$, **A**). As determined by ANOVA, the P-values of the statistical evaluations are indicated for the comparisons of healthy women *vs* patients with benign disease, healthy women *vs* breast cancer patients and patients with benign disease *vs* breast cancer patients (**B**).

Table 3. Prediction results from leave-one-out cross-validation			
	Predicted		
	Healthy	**Benign**	**Malignant**
True status			
Healthy	22	3	2
Benign	4	7	8
Malignant	2	1	65

cancer patients after surgery than in patients before surgery ($P < 0.001$, Figure 2).

The cohort of 34 patients was too small to result in potential associations of circulating mRNA and microRNAs with the clinicopathological parameters or different treatment arms. The mastectomy had no influence on the yields of circulating RNA and miRs.

Association of miR-202 with overall survival of cancer patients. We compared the log2 transformed concentrations of circulating RNA and miRs in the blood serum of 102 breast cancer patients with their clinical and histopathological data (Table 1). For these statistical evaluations, t-test or ANOVA were used where appropriate. No significant correlations were found after correction for multiple testing.

To assess whether the serum levels of circulating RNA and miRs were related to patient outcome (overall survival, OS; disease-free survival, DFS), Kaplan–Meier and log-rank models, as well as univariate and multivariate Cox regression models were carried out. The mean follow-up time of the cancer patients was 6.2 years (range 0.6–11.9 years). The REMARK criteria for prognostic studies (McShane *et al*, 2006) have been taken into consideration. Median values of RNA and miRs were used for grouping the serum samples according to low and high levels. As shown in Figure 3, higher serum concentrations of miR-202 (log-rank test: $P = 0.004$, Cox: $P = 0.0001$) significantly correlated with poor OS. The mean OS periods were 139 and 110 months (95% CI 129–149 and 96–125) in patients who had miR-202 concentrations of < 0.3 and $> 0.3 \, \text{ng ml}^{-1}$ serum, respectively (Figure 3A). Based on these significant results of miR-202, we also performed univariate OS analyses with the postoperative miR-202 concentrations. Although the number of 34 postoperative samples is small, we detected that high serum levels of miR-202 after surgery correlated with decreased OS (log-rank test: $P = 0.063$, Cox: $P = 0.022$, data not shown). With the exception of a tendency of correlation of BORIS RNA concentrations with patient prognosis ($P = 0.071$), no further significant associations were found. In addition, there was no correlation of the serum levels of circulating RNA and miRs with DFS.

Furthermore, we evaluated the correlation of the levels of miR-202 and BORIS RNA with patient prognosis in subsets of patients grouped according to clinicopathological parameters. MiR-202 concentrations showed prognostic significance in several
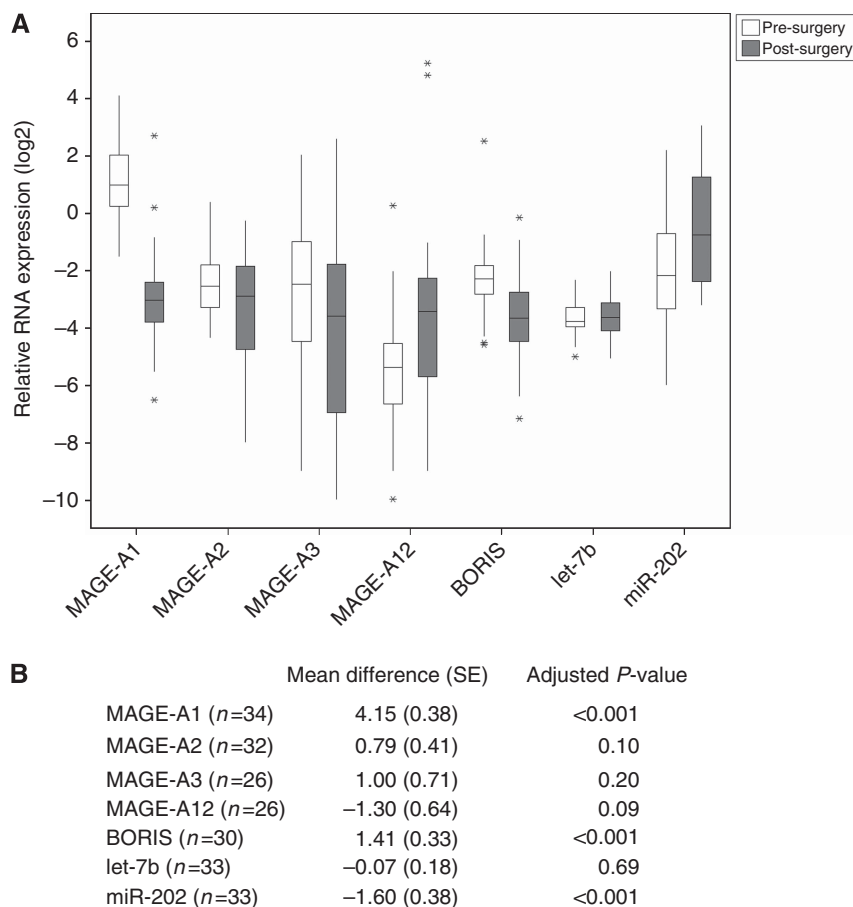
B	Mean difference (SE)	Adjusted *P*-value
MAGE-A1 (*n*=34)	4.15 (0.38)	<0.001
MAGE-A2 (*n*=32)	0.79 (0.41)	0.10
MAGE-A3 (*n*=26)	1.00 (0.71)	0.20
MAGE-A12 (*n*=26)	−1.30 (0.64)	0.09
BORIS (*n*=30)	1.41 (0.33)	<0.001
let-7b (*n*=33)	−0.07 (0.18)	0.69
miR-202 (*n*=33)	−1.60 (0.38)	<0.001

Figure 2. Serum levels of RNA and miRs in breast cancer patients before and after surgery. Box plots showing the comparison of MAGE-A1, -A2, -A3, -A12 and BORIS RNA, and let-7b and miR-202 levels in serum of healthy individuals ($n = 37$), patients with benign breast disease ($n = 26$) and breast cancer patients before ($n = 34$) and after surgery ($n = 34$, **A**). As determined by ANOVA, the mean differences and adjusted *P*-values of the statistical evaluations are indicated (**B**).

subgroups (Figures 3B–D). In patients with lymph node metastases ($P = 0.008$, log-rank test, Figure 3B), tumour stages pT2-4 ($P = 0.03$, Figure 3C) and patients with absence of disseminated tumour cells in bone marrow, ($P = 0.017$, Figure 3D) elevated miR-202 concentrations significantly correlated with poor OS. In patients with lymph node metastases, who had miR-202 concentrations above the median, OS was 93 months compared to 131 months in those with levels below median ($P = 0.008$, Figure 3B). Likewise, in this patient subgroup OS was also shorter, when the patients had elevated BORIS concentrations, with a mean of 81 months compared to 134 months in patients with levels below median ($P = 0.003$, Figure 3E). In exploratory multivariate OS analysis (Cox regression models), we could only include a variable that was significantly relevant to patient prognosis. With the exception of nodal status ($P = 0.047$), the other clinicopathological parameters did not significantly correlate with OS in our patient cohort performing univariate analyses. High serum levels of miR-202 proved to be an independent prognostic factor in uni- and multivariate analyses ($P = 0.0001$).

Let-7b and miR-202 have no effect on the protein expression of MAGE-A1 in MCF-7 and BCM1 cells. The screening of the miRbase databases (Griffiths-Jones *et al*, 2006) revealed potential binding sites of let-7b and miR-202 in the 3′UTR of MAGE-A1 mRNA. To examine whether expression of MAGE-A1 is regulated by these miRs, we performed transfections of the cell lines MCF-7 and BCM1 using mimics or inhibitors of let-7b and miR-202 and scrambled RNA as a negative control. The mimics are double-stranded RNA molecules that mimic the mature endogenous let-7b

and miR-202, whereas the inhibitors are single-stranded, modified RNA molecules, which after transfection, specifically binds to mimics and endogenous let-7b and miR-202 and inhibits their function. The data of quantitative real-time PCR using MAGE-A1-specific primers (data not shown) and of western blotting using antibodies specific for MAGE-A1 (Figure 4) showed no effect of the mimics and inhibitors on the RNA and protein levels of MAGE-A1, respectively. On the western blot the signals derived from MCF-7 cells transfected with mimics and inhibitors of both miRs (let-7b and miR-202) and scrambled control as well as from basal (non-transfected) cells were similar (Figure 4) pointing to the hypothesis that both miRs do not bind to the 3′UTR in the MAGE-A1 mRNA.

DISCUSSION

In the present study we quantified the RNA levels of MAGE-A1, -A2, -A3, -A12 and BORIS as well as of the microRNAs let-7b and miR-202 in the serum of breast cancer patients, patients with benign breast disease, and healthy women. Our analyses show that the serum levels of MAGE-A1, -A2, -A3 and BORIS RNA as well as of let-7b could differentiate between malignant and benign breast tumours. After surgery a decrease in the circulating transcript levels of MAGE-A1 and BORIS and an increase in the levels of miR-202 were observed. The key finding of our study is that in uni- and multivariate analyses high serum levels of miR-202 proved to be an independent prognostic factor.
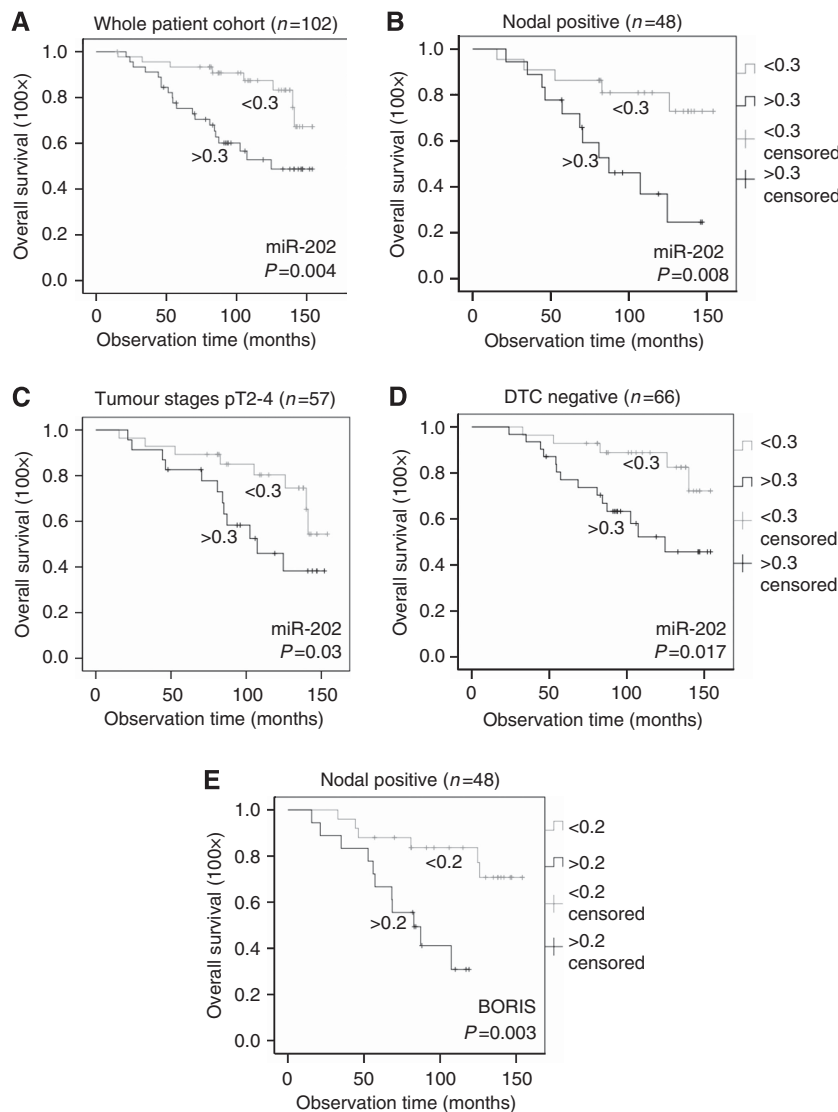
Figure 3. Serum levels of miR-202 and BORIS significantly correlate with overall survival. Univariate Kaplan–Meier survival curves related to concentrations of circulating miR-202 and BORIS in breast cancer patients. Overall survival is significantly associated with serum miR-202 levels in the whole patient cohort (**A**, $n=102$), in the subgroups of nodal-positive patients (**B**, $n=48$), patients with tumour stages pT2-4 (**C**, $n=57$) and patients without DTCs in their bone marrow (**D**, $n=66$). Overall survival is also associated with serum BORIS levels in the subgroup of nodal-positive patients (**E**, $n=48$). For miR-202 and BORIS median values of 0.3 and 0.2 were used for grouping the serum samples according to low and high levels, respectively.

First, we examined the mRNA expression of MAGE-A1, -A2, -A3 and -A12 and BORIS as well as let-7b and miR-202 in a variety of cancer cells. MAGE-A1, -A2 and -A3, and BORIS were expressed in most cell lines. Whereas let-7b was expressed in each cell line, low levels of miR-202 could only be detected in one cell line. There was no positive and negative correlation of MAGE-A transcripts with BORIS mRNA and both miRs, respectively. The expression of MAGE-A genes in BORIS-negative cancer cells could be explained either by the used primer pair, which does not amplify all splice variants of BORIS or in case of high levels of MAGE-A1 expression by complete demethylation of its promoter – thus, by stabilisation of epigenetic changes. The latter is well supported by data on the inability of BORIS to further increase MAGE-A1 expression, if it is already highly expressed (Vatolin et al, 2005).

MAGE-A and their epitope peptides constitute important targets for anti-tumour immunotherapy. Their strict expression on the surface of malignant cells has led to several immunotherapeutic trials targeting some of these members (Sang et al, 2011; Meek and Marcar, 2012). Thus, the detection of the elevated levels of MAGE-A1, -A2 and -A3 in the blood of breast cancer patients could become a blood-based tool for early cancer diagnosis and a potential predictive biomarker for MAGE-directed immunotherapies. In contrast, the levels of MAGE-A12 transcripts in the blood of breast cancer patients seem to be of no diagnostic value.

Our observation supports the hypothesis that a part of the cell-free RNA in the blood of cancer patients originates from cancer cells and that these RNA molecules seem to circulate stably in blood. Circulating RNA could, therefore, withstand RNA degradation factors because most of them are included in apoptotic bodies, microvesicles, or exosomes (Orozco and Lewis, 2010). Besides apoptotic and necrotic cell death, living cancer cells may also actively release RNA into the blood circulation (Stroun et al, 2001).

Apart from a marginal correlation of increasing serum levels of MAGE-A2 with lymph node metastases, the serum levels of MAGE-A and BORIS mRNA were not associated with the clinicopathological parameters of breast cancer patients in our cohort. Our findings suggest that these serum factors are not qualified for detection of cancer progression, but may represent concomitant features in the
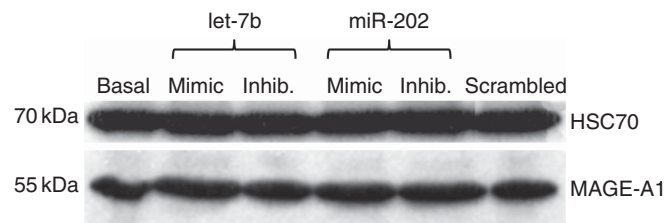
Figure 4. Let-7b and miR-202 do not affect the protein expression of MAGE-A1. Protein levels in MCF-7 cells were analysed by western blot. HSC70 signals served as a loading control. MAGE-A1 protein levels in MCF-7 cells transfected with only transfection agent (basal) or transfected with mimics and inhibitors (Inh.) of let-7b and miR-202 or scrambled RNA (negative control). Representative analysis of three independent experiments is shown, all experiments were performed in duplicate.

process of cellular transformation related to the global genome hypomethylation (Feinberg and Tycko, 2004).

Surprisingly, we also found elevated levels of MAGE-A1, -A2 and BORIS in the blood of patients with benign breast disease albeit they were significantly lower than the levels in cancer patients. In contrast to our data, Kwon *et al* detected no MAGE-A gene expression in patients with benign disease and in their cohort more patients with positive lymph node status and metastatic disease had MAGE-A gene expression than patients with primary cancer. Their findings suggested that the detection of MAGE-A gene expression in the blood may be cancer-specific and predict tumour progression or recurrence (Kwon *et al*, 2005). This discrepancy to our data can be explained that their study used whole blood (and not serum) samples and nested PCR, a technique which is less sensitive and does not quantify the MAGE-A amounts.

As sequence analyses suggested binding sites of the microRNAs let-7b and miR-202 in the 3′UTR of MAGE-A1 RNA, we additionally quantified the levels of circulating microRNAs let-7b and miR-202. The let-7 family, which let-7b and miR-202 belongs to, regulates oestrogen receptor alpha signalling in oestrogen receptor-mediated cellular malignant growth of breast cancer (Zhao *et al*, 2011), and is involved in self-renewal and tumour-igenicity of breast cancer cells (Yu *et al*, 2007). The members are often reported as tumour suppressors and associated with various cancer types (Wang *et al*, 2012). Therefore, it was surprising to find the serum levels of let-7b and miR-202 to be upregulated in our cohort of breast cancer patients. However, similar to our study elevated levels of these miRs in the blood circulation of breast and ovarian cancer patients have also been described (Cookson *et al*, 2012; Tang *et al*, 2013). In the present study, our uni- and multivariate analyses showed that the increased serum concentrations of miR-202 may be a strong independent prognostic factor for breast cancer patients. The miR-202 levels were also of prognostic significance in the subgroups of patients with lymph node metastases and of advanced tumour stages. To our knowledge, this is the first report on the prognostic impact of circulating miR-202 in breast cancer. To date, only one study has evaluated the levels of circulating miR-202 in the blood of breast cancer patients. This study showed that miR-202 was significantly upregulated in whole blood samples of early-stage breast cancer patients performing microarray and quantitative PCR (Schrauder *et al*, 2012).

To examine the impact of both miRs on the protein expression of MAGE-A1, we performed transfection studies using mimics and inhibitors of let-7b and miR-202 and western blot analyses using a MAGE-A1-specific antibody. Both miRs had no effect on the protein expression of MAGE-A1. Our findings suggest that these miRs do not bind to the 3′UTR in the MAGE-A1 RNA. To confirm this hypothesis, we tried to clone the binding region of the MAGE 3′UTR, three times in series, into the pmiRGlo vector

(reporter plasmid). As previously found, the impact of a miR on the repression of its target gene is hardly measurable if plasmids containing only one binding site are used in transfection assays. However, the cloning of three sequences in series into the plasmid was impeded by technical problems. Moreover, we carried out transfections assays using reporter plasmids containing the MAGE-A1 promoter (Wischnewski *et al*, 2006), and the mimics of let-7b and miR-202, because there is growing evidence that miRs can also serve as activators of gene expression by targeting gene regulatory sequences. Target sites for miRs have been found in gene promoters, and these complementary sequences were as common as those within the 3′UTR of mRNAs (Portnoy *et al*, 2011). However, our preliminary data showed that both miRs have no influence on the promoter activity of MAGE-A1. Our findings suggest that let-7b and miR-202 do not seem to be involved in the regulation of MAGE-A1 protein expression.

Finally, we compared the pre- and postoperative serum levels of MAGE-A mRNA, BORIS mRNA and miRs. The significant decline of postoperative serum levels of MAGE-A1 and BORIS mRNA might be a consequence of resection of the tumours 2–3 months before taking the blood samples for our analysis. Moreover, it can only be speculated why the levels of MAGE-A2, MAGE-A3, let-7b and miR-202 did not decrease after surgery. Here, inflammatory processes and active secretion could contribute to discharge these molecules. Besides, we cannot exclude other tissues, such as circulating tumour cells or occult micrometastases, to be sources of the postoperative serum levels. Conspicuously, the levels of miR-202 even postoperatively increased. Although the number of postoperative samples was small and the statistical evaluation has to be critically considered, we detected that the increased postoperative serum levels of miR-202 also correlated with poor OS. These findings suggest that the concentrations of circulating miR-202 after surgery seem to be of prognostic value, too.

Based on their high immunogenicity, significant effort has been made to develop antibodies and cancer vaccines towards MAGE-A. MAGE-A-targeted approaches could inhibit or even eliminate MAGE-A cancer-promoting activities in human disease. In respect to their predominant expression in germ cells, such therapies may offer a high degree of specificity with a minimum of potential adverse effects. The widespread expression of MAGE-A antigens in different cancer entities means that therapies may not be only applicable to breast cancer, but also to many other cancer types. Our findings show the cancer-specific increase in the serum levels of MAGE-A, BORIS and let-7b in breast cancer patients. The tumour-specific increases in serum concentrations of miR-202 are an independent marker capable of predicting high-risk patients with poor overall survival and could assist in individualised management and monitoring of those patients. However, large prospective clinical studies are warranted to confirm our results and further explore the potential of circulating MAGE-A as diagnostic and therapeutic breast cancer serum markers for breast cancer patients. Currently, studies are being carried out to investigate the target mRNAs of let-7b and miR-202.

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