Regulatory BC200 RNA in peripheral blood of patients with invasive breast cancer

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ABSTRACT

Regulatory brain cytoplasmic 200 RNA (BC200 RNA) is highly expressed in human mammary carcinoma cells. Here, we ask whether BC200 RNA becomes detectable in peripheral blood of patients with invasive breast cancer. Using quantitative reverse-transcription PCR (qRT-PCR) methodology, we observed that BC200 RNA blood levels were significantly elevated, in comparison with healthy subjects, in patients with invasive breast cancer prior to tumorectomy (p=0.001) and in patients with metastatic breast cancer (p=0.003). In patients with invasive breast cancer who had recently undergone tumorectomy, BC200 RNA blood levels were not distinguishable from levels in healthy subjects. However, normality analysis revealed a heterogeneous distribution of patients in this group, including a subgroup of individuals with high residual BC200 RNA blood levels. In blood from patients with invasive breast cancer, BC200 RNA was specifically detected in the mononuclear leukocyte fraction. The qRT-PCR approach is sensitive enough to detect as few as three BC200 RNA-expressing tumor cells. Our work establishes the potential of BC200 RNA detection in blood to serve as a molecular indicator of invasive breast malignancy.

Significance of this study

What is already known about this subject?

- Breast cancer is a leading cause of death from malignancies in women.
- Regulatory brain cytoplasmic 200 (BC200) RNA is expressed at high levels in breast cancer cells.
- Circulating tumor cells (CTCs) have been advanced as predictors of recurrent or metastatic disease.

What are the new findings?

- Quantitative reverse-transcription PCR methodology was used to establish the potential of a BC200 RNA-based blood test for invasive breast cancer.
- In patients with invasive breast cancer, BC200 RNA can be detected in peripheral blood with high specificity and sensitivity.
- Detection of BC200 RNA is a CTC surrogate marker of high discriminating power.

How might these results change the focus of research or clinical practice?

- BC200 RNA detection in blood is a minimally invasive and highly discriminating molecular indicator of invasive breast malignancies. High residual BC200 RNA blood levels after treatment can serve as an indicator of high relapse potential.

INTRODUCTION

Neuronal BC RNAs, an abundant subtype of small cytoplasmic RNAs, are translational regulators that operate in the translational control of gene expression in mammalian nerve cells.[1 2] BC RNAs interact with components of the eukaryotic translational machinery. Specifically, primate BC200 RNA and rodent BC1 RNA target eukaryotic initiation factors (eIFs) 4A and 4B, canonical translation factors that are required for the recruitment of small ribosomal subunits to mRNAs with higher-order structure content in their 5′ untranslated regions.[2–6] While BC RNAs act as translational repressors in the basal default state, BC RNA control can reversibly switch from repressive to permissive on receptor activation in neurons.[6] Specifically, stimulation of group I metabotropic glutamate receptors causes rapid activation of protein phosphatase 2A (PP2A), which in turn triggers dephosphorylation of eIF4B at serine 406 (S406). Thus dephosphorylated, eIF4B interacts with BC RNAs eIF4B at significantly reduced affinity, as a result allowing the factor to engage the small ribosomal subunit for translation initiation.[6]

BC RNAs are not typically expressed in somatic mammalian cell types other than neurons.[2 7] However, in a notable exception to this neuronal specificity, BC200 RNA was found expressed at high levels in human invasive breast carcinoma cells.[8 9] In contrast, BC200 RNA was not detected in normal human breast epithelial cells or in benign tumors of the breast. The combined data suggested that regulatory BC200 RNA operates in invasive or preinvasive breast carcinoma cells where it causes dysregulation of cellular translational control.[9 10]

Despite the fact that a combination of factors, including early detection (eg, mammography) and improved therapeutic intervention (resection,

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adjuvant therapy), has in recent years resulted in a decrease of breast cancer mortality in the USA, breast cancer remains a leading cause of death from malignancies in women.[11] However, mammography has been a subject of controversial debate,[12–15] and therapeutic intervention has been hampered by a lack of indicators, molecular and otherwise, that would aid physicians and patients in breast cancer treatment decisions. In particular, any improvement in our ability to predict treatment outcome, and to prognosticate tumor progression and recurrences, would have potentially significant clinical use.

Here, we hypothesized that invasive breast cancers may disseminate BC200-expressing cells into the circulation. Considering that BC200 RNA (1) is a small regulatory RNA with significant structural stability[16] and (2) is expressed at high levels in breast cancer cells,[8 9] we further hypothesized that the RNA may be persistently expressed in circulating tumor cells (CTCs) derived from invasive breast cancers. The goal of the present study was to test this hypothesis, using reverse-transcription (RT)-PCR and quantitative RT-PCR (qRT-PCR) methodology, and to ascertain the potential of an RNA-based blood test for invasive breast cancer.

MATERIALS AND METHODS

Subject recruitment

Peripheral blood was collected from the following subject groups:

1. Healthy subjects: This group comprised apparently healthy women without diagnosis, or signs or symptoms, of cancer (n=12).
2. No evidence of disease: This group comprised previous patients with breast cancer who had completed treatment, including adjuvant therapy, at least 1.5 years prior to blood collection. These patients were under surveillance, with no evidence of current disease, at the time of blood collection (n=10).
3. Primary disease I: This group comprised patients with operable breast cancer after diagnosis but before resection of the primary tumor (n=14).
4. Primary disease II: This group comprised patients with operable breast cancer after tumorectomy who, at the time of blood collection, were either undergoing adjuvant therapy or had recently completed adjuvant therapy (recently being defined as within 1 month before blood collection; n=12).
5. Metastatic disease: This group comprised patients with active metastatic disease, stage IV (n=23). Patient parameters are listed in table 1. Patients' records and oncological assessments were reviewed by three oncologists (EQC, RLC, and GAS).

Specimen characteristics

Blood samples were drawn from the median cubital vein on the anterior forearm and collected in BD Vacutainer tubes

Table 1 Classification of breast cancer

	No evidence of disease	Primary disease I (presurgery)	Primary disease II (postsurgery)	Metastatic disease
No of patients	10	14	12	23
Age (mean)	62.9	59.1	53.8	53.7
Premenopausal	1	5	5	8
Postmenopausal	9	9	7	15
Stage I	6	0	1	0
Stage II	3	5	7	0
Stage III	1	9	4	0
Stage IV	0	0	0	23
Tumor size 1–1.9 cm	6	0	2	1
Tumor size 2–3.9 cm	1	3	7	2
Tumor size >4 cm	1	11	3	14
Unknown	2	0	0	6
Grade 1 or 2	3	5	3	6
Grade 3	4	8	7	12
Unknown	3	1	2	5
Lymph node 0	7	3	6	4
Lymph nodes 1–3	3	11	6	15
Unknown	0	0	0	4
Estrogen receptor +	6	7	8	15
Estrogen receptor –	1	7	4	8
Unknown	3	0	0	0
Progesterone receptor +	5	7	8	10
Progesterone receptor –	2	7	4	13
Unknown	3	0	0	0
Her2/neu +	4	12	8	19
Her2/neu –	2	1	4	4
Unknown	4	1	0	0

containing EDTA (BD, Franklin Lakes, NJ). Then 250 µL aliquots were quick-frozen at –80°C. A 250 µL aliquot typically generated 1.5 µg total RNA. Furthermore, 10 ng total RNA was used per reaction, that is, 30 ng for triplicate BC200 RNA amplification and 30 ng for triplicate control RNA amplification reaction, in total 60 ng per experiment.

RNA isolation, RT-PCR, and qRT-PCR

Total RNA was extracted from 250 µL whole blood using the Ribopure Extraction Kit (Ambion, Austin, TX). RNA samples were treated with the Turbo DNA-free kit (Ambion) to minimize DNA contamination. After DNase treatment, each sample was tested for residual genomic DNA by omitting the RT reaction. Only samples that did not exhibit detectable DNA background were used in this work. The reverse transcription step was performed as follows, using random hexamer primers and SuperScript III (Thermo Fisher, Springfield, NJ): 5 min at 25°C, 1 hour at 50°C, and 15 min at 70°C. PCR amplification reactions were carried out in a final volume of 50 µL using 1.0 U of Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA). PCR amplification conditions were as follows: 1 cycle of 1 min at 94°C, 45 s at 57°C, 1 min at 72°C, followed by 37 cycles of 30 s at 94°C, 45 s at 57°C, 1 min at 72°C, and a final cycle of 1 min at 94°C, 45 s at 57°C, 15 min 72°C. Conditions for qPCR were as follows: 30 s at 95°C, 30 s at 58°C, 30 s at 72°C, carried out for 40 cycles. qPCR was performed with the IQ SYBR Green Supermix dye kit (Bio-Rad, Hercules, CA), using a CFX96 Real-Time PCR System (Bio-Rad). Primers for the amplification of BC200 RNA were as follows:

BC200 forward primer: 5′ CCTGGGCAATATAGC-GAGAC 3′

BC200 reverse primer: 5′ GGGTTGTTGCTTT-GAGGGA 3′

The predicted amplification product corresponds to nt 91–188 of the 3′ region of BC200 RNA. The 97-nt-length PCR product was sequenced and verified as corresponding to the above 3′ BC200 RNA segment.

As an internal standard for qRT-PCR, we used the mRNA encoding acidic ribosomal protein (ARP, also known as ribosomal protein P0, NM_001002), using ARP primers as described.[17] ARP mRNA was used to normalize the amount of total RNA in each qRT-PCR reaction.[18] As an independent internal control, we used primers specific for Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, SABioscience, Frederick, MD), as described by the manufacturer. qRT-PCR data were analyzed using the cycle threshold (C_t) method.[19] For each sample, the C_t for BC200 RNA was normalized to the C_t for ARP mRNA, resulting in a ΔC_t that reflects the relative level of BC200 RNA in that sample. For a blood sample from a patient with breast cancer, the ΔC_t was normalized to the mean ΔC_t obtained with samples from healthy subjects, resulting in a $\Delta\Delta C_t$ for the patient sample. $2^{-\Delta\Delta Ct}$ was then calculated as a measure of the fold increase of BC200 RNA in the patient's blood sample, relative to control blood samples from healthy subjects.

Blood fractionation

To prepare blood fractions, in particular the mononuclear leukocyte (MNL) fraction, which includes CTCs, we performed cell fractionation using the Ficoll-Paque procedure (Histopaque 1077; Sigma-Aldrich, St. Louis, MO), as described.[20] In brief, blood samples were diluted with phosphate buffered saline (PBS) (without Ca^{2+} or Mg^{2+}) and were layered on 3 mL of a Ficoll gradient. After centrifugation at 300×*g* for 10 min, MNL, RBC/PML (red blood cell/polymorphonuclear leukocyte fraction), and plasma fractions were collected, washed with PBS, and RNA-extracted using Ribopure.

Limiting dilution experiments with MCF-7 cells

MCF-7 cells (ATCC, Manassas, VA) were used as a breast cancer cell system. For limiting dilution experiments, we used cell lysates to avoid random variations in the number of cells included in the assay. Lysates of known numbers of MCF-7 cells were prepared using lysis buffer (10 mM glycine, 1% Triton X-100). A given amount of MCF-7 cell lysate, representing the desired number of cells, was then added to total RNA isolated from 10^4 Baby Hamster Kidney (BHK) cells or to 10 ng of total RNA isolated from blood of healthy subjects. BC200 RNA levels in MCF-7 cells were established against the same amount of total RNA from the blood of healthy subjects that was used in figure 1. BHK cells were obtained from ATCC. Total RNA was prepared using TRIzol (Invitrogen). Samples were DNase-digested and reverse transcribed as described above.

Expression of BC200 RNA in tumor cell lines

MCF-10A, MCF-7, BHK, and HeLa cells were collected and washed with PBS. Total RNA was extracted with TRIzol (Invitrogen). Reverse transcription and qPCR amplification reactions were carried out using the same reagents, primers, and amplification conditions as described above. qRT-PCR results were analyzed using the cycle threshold (C_t) method (for details, see previous section).

Statistical methods

Statistical analysis was performed on 71 blood samples in five groups. To analyze the significance of BC200 RNA blood levels among groups, we used non-parametric Kruskal-Wallis one-way analysis of variance by ranks. The non-parametric Mann-Whitney U-test was used for comparisons of two groups. Receiver operating characteristic (ROC) analysis was used to establish discriminative power of BC200 RNA as a diagnostic and prognostic indicator. In ROC analysis, sensitivity is plotted against (1−specificity).[21] The area under the curve (AUC) is an index of the discriminative diagnostic power of a given test, with an ideally performing test having an AUC of 1.0. Statistical analysis was performed using SPSS software.

RESULTS

BC200 RNA in blood of patients with breast cancer

We initially used RT-PCR to establish whether BC200 RNA is detectable in peripheral blood of patients with invasive breast cancer. RT-PCR amplification revealed that BC200 RNA levels in blood from a patient with breast cancer with metastatic disease were substantially higher than those from a healthy subject (figure 1A).

Subsequently, we collected peripheral blood from a total of 71 subjects (Materials and methods section).

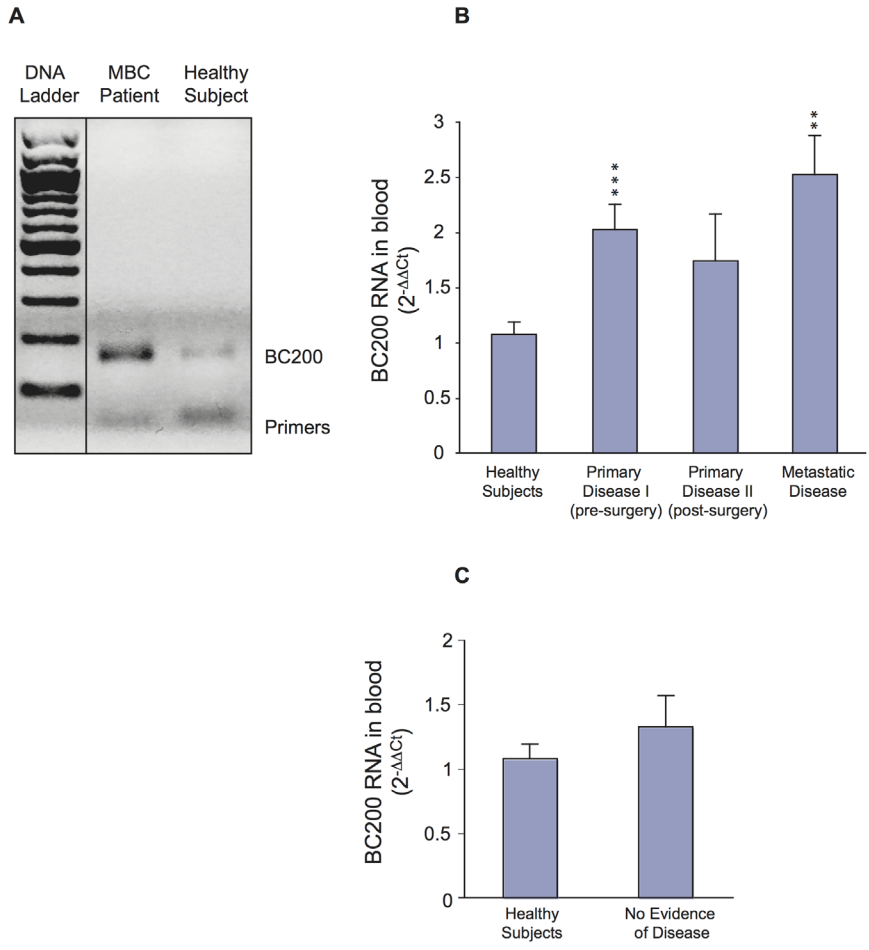

Figure 1 BC200 RNA blood levels in patients with breast cancer. (A) High levels of BC200 RNA are detected in peripheral blood from a patient with metastatic breast cancer (MBC). Total RNA was isolated from whole blood. The RT-PCR product was resolved on a 1.2% agarose gel (bands at the bottom of the gel represent primers used in the PCR reaction). Medical history: The patient was diagnosed with breast cancer 2 years prior to BC200 RNA analysis. She underwent surgery and adjuvant therapy, including six cycles of doxorubicin and cyclophosphamide followed by anastrozole (Arimidex). Two years later, she was diagnosed with bone metastases and was scheduled to undergo a new treatment cycle with paclitaxel (Taxol) and trastuzumab (Herceptin). Blood for BC200 RNA analysis was drawn after diagnosis of recurrent disease but before Taxol/Herceptin treatment. It appears that in this case, that is, prior to the initiation of adjuvant therapy, BC200 RNA blood levels are higher than average BC200 RNA blood levels in metastatic disease. (B) Bar diagram shows qRT-PCR analysis of BC200 RNA expression levels in whole blood from healthy subjects and from three groups of patients with breast cancer (presurgery, postsurgery, metastatic disease). BC200 RNA amplification data were normalized to ARP mRNA.[17 18] Data are shown in the format mean±SEM, with each data point representing three experiments for each blood sample analyzed. BC200 RNA blood levels are shown as fold increase in comparison with the control group (healthy subjects), calculated as $2^{-\Delta\Delta Ct}$ (Materials and methods section).[19] Statistical analysis: non-parametric Kruskal-Wallis analysis (p=0.005) followed by non-parametric Mann-Whitney U-test; **p=0.003, ***p=0.001. (C) BC200 RNA blood levels in former patients with breast cancer with no evidence of recurrent or residual disease are similar to those in healthy subjects. Statistical analysis: Kruskal-Wallis (p=0.668).

BC200 RNA levels were established in blood samples from these subjects by qRT-PCR. The results are shown in diagrammatic form in figure 1B,C. High levels of BC200 RNA were detected in blood samples from patients with breast cancer with active disease. BC200 RNA blood levels were significantly elevated in patients with either primary or metastatic disease, in comparison with healthy subjects (figure 1B). Specifically, patients with breast cancer with primary disease who had not yet undergone tumorectomy exhibited a substantial increase of BC200 RNA levels (twofold in comparison with healthy subjects; figure 1B, group Primary Disease I). This increase was highly significant (***p=0.001, Mann-Whitney U-test), suggesting high discriminating power. Patients with metastatic disease (figure 1B, group Metastatic Disease) exhibited an almost two-and-a-half-fold average increase of BC200 RNA blood levels, in comparison with healthy subjects (**p=0.003, Mann-Whitney U-test).

In postsurgery patients (figure 1B, group Primary Disease II), BC200 RNA blood levels had decreased and were not

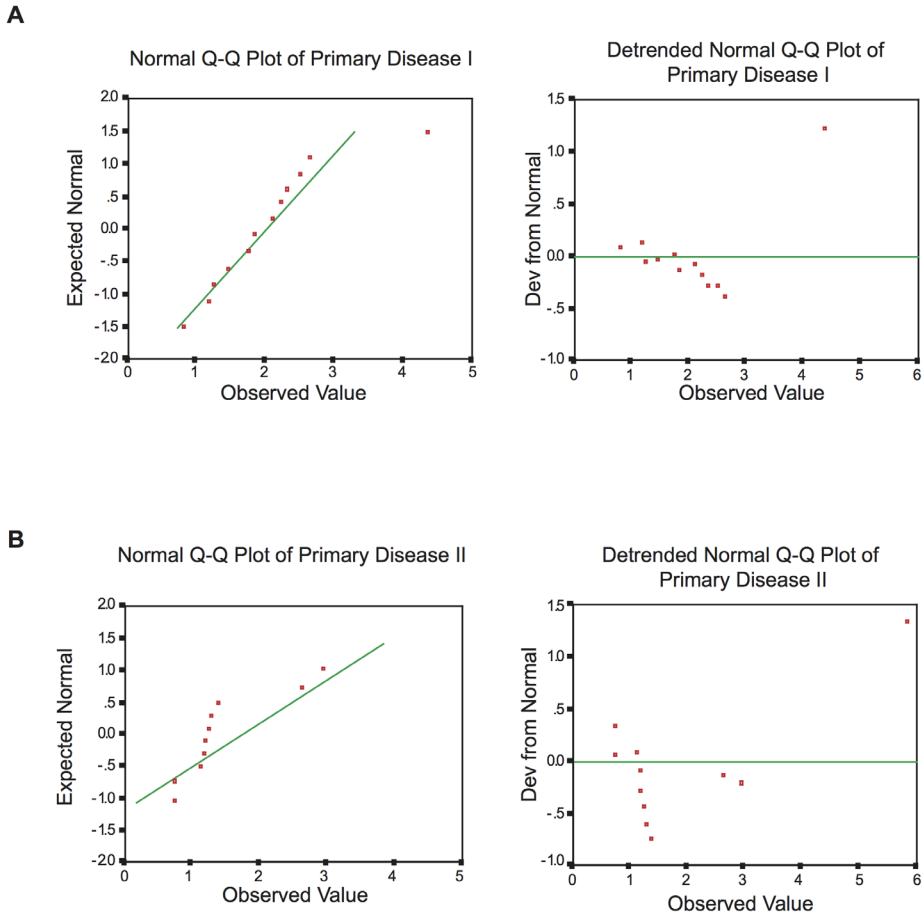

Figure 2 Sample distribution analysis of Primary Disease I and Primary Disease II patient groups (normal and detrended normal Q-Q plots). Normality analysis of Primary Disease I (primary disease patients prior to tumorectomy) sample distribution shows no significant deviation from normal distribution (green line, A). Conversely, analysis of Primary Disease II group (postsurgery primary disease patients) sample distribution shows a significant departure from normality (green line, B). Kolmogorov-Smirnov, $p<0.0005$.

significantly different from BC200 RNA blood levels in healthy subjects. This result is consistent with the notion that BC200 RNA in peripheral blood of patients with invasive breast cancer (ie, group Primary Disease I) is contributed by CTCs that have been released from the primary tumor, and that further release of cells would cease after tumor resection. Similar observations have been reported after excision of primary malignancies other than breast cancer.[22] However, BC200 RNA blood levels of the postsurgery patient group were also not significantly different from levels of the presurgery patient group. The data indicate that in postsurgery patients, BC200 RNA is detected in blood at levels intermediate between presurgery patients and healthy subjects. In addition, the SEM in postsurgery group is relatively high, an indication of heterogeneity in BC200 RNA blood levels in this patient group. It is possible that such heterogeneity is a reflection of varying treatment success, for example, a relatively high number of residual CTCs in patients with high blood levels of BC200 RNA.

The above considerations were confirmed by normality analysis. Normal Q-Q plots and detrended normal Q-Q plots show that BC200 RNA levels in the Primary Disease I group (figure 2A) conform to a normal distribution (shown in green). In contrast, BC200 RNA levels in the Primary Disease II group (postsurgery primary disease breast cancer patients) displayed a marked departure from normal distribution (figure 2B). Kolmogorov-Smirnov analysis of normality indicated a high significance of this deviation ($p<0.0005$), a result consistent with a heterogeneous population distribution. The skewness for this patient group was 2.3, a likely reflection of the presence within this group of a subgroup of individuals with high residual BC200 RNA blood levels. We note that some intragroup heterogeneity was observed for BC200 RNA blood levels in all patient groups, but for groups other than postsurgery primary disease patients, such deviation from normality did not reach statistical significance (Kolmogorov-Smirnov, $p>0.2$). The combined data indicate heterogeneity of BC200 RNA blood levels among postsurgery patients, a heterogeneity that likely reflects, within this group of patients, the presence of subgroups with high versus low residual BC200 RNA levels.

We also examined blood samples from former patients with breast cancer with no evidence of residual disease. These patients had been undergoing surveillance for several years post-treatment and were considered disease free at

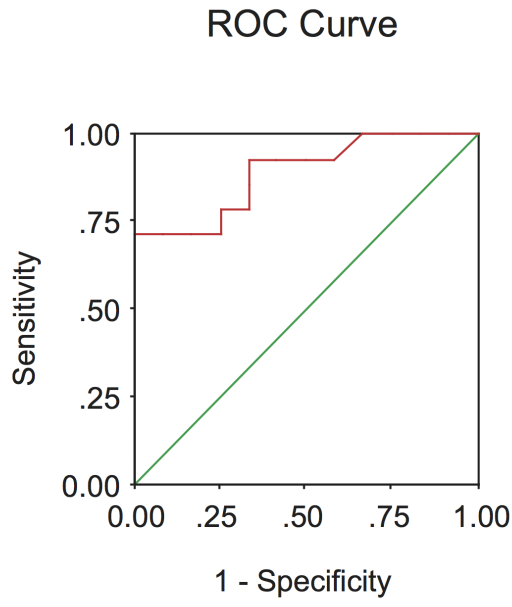

Figure 3 ROC analysis of BC200 RNA expression in peripheral blood of patients with invasive breast cancer. To ascertain the discriminative diagnostic power of the BC200 RNA blood test, receiver operating characteristic (ROC) analysis was performed by plotting sensitivity against (1−specificity), comparing BC200 RNA levels in blood from healthy subjects with BC200 RNA levels in presurgery patients with invasive breast cancer (group Primary Disease I). The obtained ROC curve shows an area under the curve of 0.89 (95% CI of 0.77 to 1.0), indicating a high discriminative efficacy of the BC200 RNA test.

the time of blood sample collection. In blood samples from these patients, BC200 RNA levels were significantly lower than in samples from patients with active disease, primary or metastatic, and were not significantly different from BC200 RNA levels in samples from healthy subjects (figure 1C).

Patient tumor characteristics are listed in table 1. Characteristics are variable among patient groups, and such variability could potentially impact BC200 RNA expression levels. It is noted that while previous work has established significant differences in BC200 RNA tissue levels between patients with invasive breast cancer and healthy subjects, no significant differences in such levels were observed between patients with breast cancer with different subtypes of invasive disease.[9]

Discriminative diagnostic power

ROC analysis was performed to calculate the diagnostic efficacy of BC200 RNA blood levels as a molecular indicator of invasive breast cancer, that is, the ability of the test to correctly classify subjects with and without disease. In ROC analysis, sensitivity is plotted against (1−specificity), and the AUC is used as an index of diagnostic power, varying from 0.5 (no diagnostic power) to 1.0 (perfect diagnostic power).[21] Comparing the presurgery invasive breast cancer group (Primary Disease I) with the healthy subjects group, an AUC of 0.89 (with a 95% CI of 0.77 to 1.0) was obtained for BC200 RNA blood levels (figure 3). These data indicate high discriminative power of BC200 RNA in peripheral blood as a molecular indicator of invasive breast cancer. They are in agreement with the observed lack of false positives in the healthy subject group (ie, BC200 RNA levels in all cases below mean±SEM of Primary Disease I group). Analogously, an AUC of 0.81 (95% CI 0.67 to 0.95) was obtained when comparing the metastatic disease group with the healthy subjects group, again indicating high discriminating diagnostic power (data not illustrated).

It is noted that the above ROC analyses apply to the presurgery patients as BC200 RNA blood levels were not analyzed for detection of residual disease in patients following tumor resection.

Table 2 Expression of BC200 RNA in the mononuclear cell fraction of peripheral blood of patients with breast cancer

Blood fraction	BC200 level ($2^{-\Delta Ct}$±SEM)	Student's t-test
MNL	2.24±0.56	p<0.05
PML/RBC	nd	–
Plasma	nd	–

Total RNA was isolated after cell fractionation, and qRT-PCR was performed with three fractions: MNLs, containing CTCs, PMLs, and RBCs, and plasma. BC200 RNA levels were elevated in the MNL cell fraction from patients with breast cancer, compared with the MNL cell fraction from healthy subjects. In PML/RBC fractions, BC200 RNA was not reliably detectable (nd) by qRT-PCR. BC200 RNA was also not detected in plasma samples. $2^{-\Delta\Delta Ct}$ represents the fold increase of BC200 RNA levels in a patient's blood sample, relative to blood samples from healthy subjects.
BC200 RNA, brain cytoplasmic 200 RNA; CTC, circulating tumor cell; MNL, mononuclear leukocyte; PML, polymorphonuclear leukocyte; RBC, red blood cell.

BC200 RNA levels in cellular fractions from peripheral blood

The data obtained are consistent with the notion that cancer cells expressing BC200 RNA enter the circulation as CTCs. If so, BC200 RNA would be expected to become detectable in the white blood cell fraction (ie, MNL). To test this hypothesis, we performed Ficoll fractionation of blood from patients with invasive breast cancer. We separated three blood fractions: plasma, MNL (also called buffy coat), and RBC, a fraction containing also PML.

BC200 RNA levels in MNL fractions from patients with breast cancer were increased by more than twofold in comparison with MNL fractions from healthy donors (table 2).

In PML/RBC fractions (containing mainly anucleate erythrocytes), BC200 RNA could not be reliably detected in samples from either patients with breast cancer or healthy subjects (table 2). BC200 RNA was also not detectable in plasma fractions from patients with breast cancer, a result to suggest that BC200 RNA is not released into the circulation in acellular form, that is, as 'free RNA'. Thus, BC200 RNA is reliably detected in MNL fractions derived from peripheral blood of patients with invasive breast cancer. These data suggest that BC200 RNA levels in peripheral blood are in fact reflecting the presence of CTCs that express the RNA.

Sensitivity of BC200 RNA detection in blood

To address the question of sensitivity (ie, the minimum number of cancer cells that we can reliably detect), we performed limiting dilution experiments using MCF-7

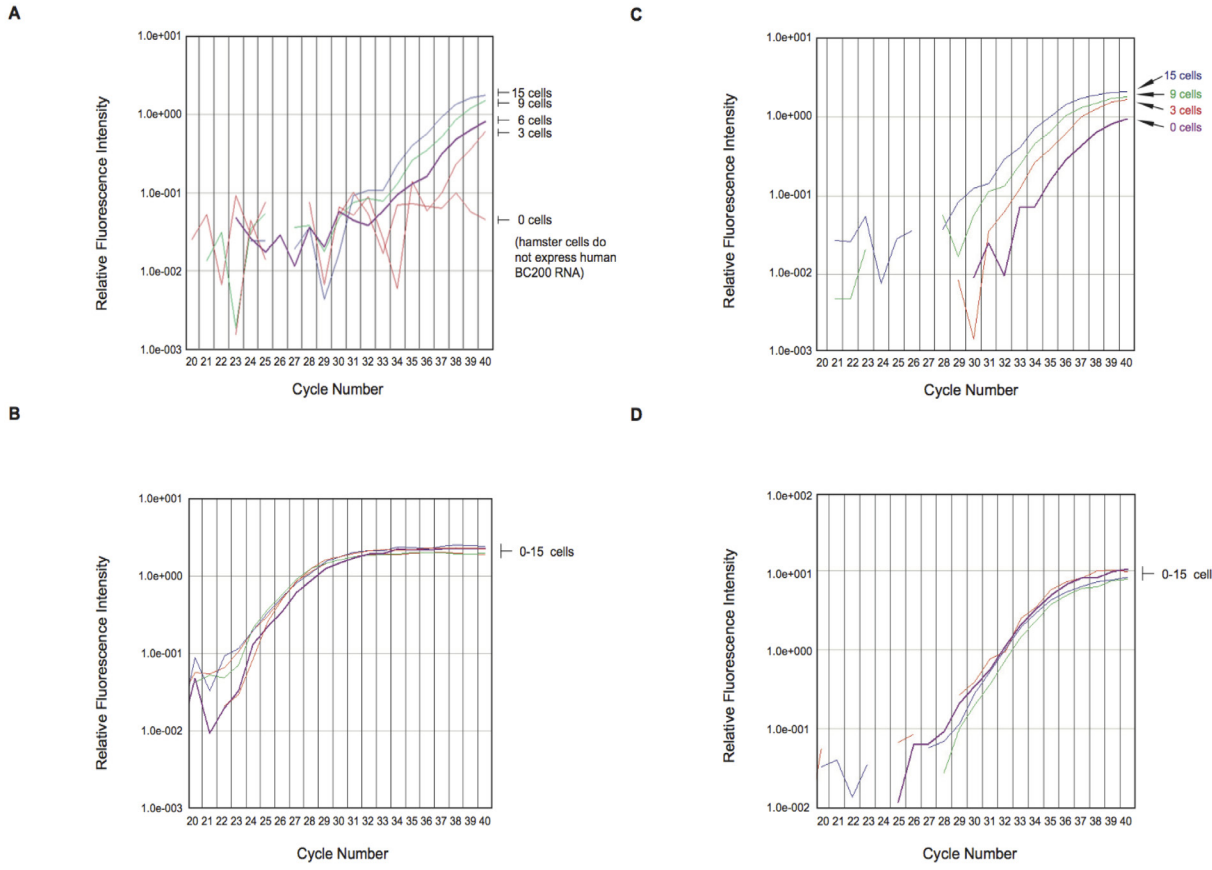

Figure 4 (A and B) Limiting dilution experiments with MCF-7 breast cancer cell RNA in Baby Hamster Kidney (BHK) cell RNA. (A) The panel shows qRT-PCR amplification plots for BC200 RNA. Total RNA isolated from MCF-7 cells, representing the respective number of cells indicated, was diluted with total RNA from 10^4 BHK cells and subjected to qRT-PCR amplification. The curves show that BC200 RNA amplification allowed detection of as few as three MCF-7 cells. The zero MCF-7 cells sample failed to reach amplification threshold after 40 cycles, indicating that no BC200 RNA was detected. (B) Control amplification experiments were performed with acidic ribosomal protein (ARP) mRNA. In contrast to BC200 amplification, ARP amplification resulted in plots that were grouped together, indicating constant expression levels independent of MCF-7 cell numbers. (C and D) Limiting dilution experiments: MCF-7 breast cancer cell RNA in RNA from blood of healthy subjects. (C) The panel shows qRT-PCR amplification plots for BC200 RNA. Total RNA isolated from MCF-7 cells (from 3 to 15 cells) was diluted with total RNA (10 ng) from blood of healthy human subjects. BC200 RNA qRT-PCR amplification allowed detection of three MCF-7 cells. (D) Control amplification experiments were performed with GAPDH mRNA. As in the experiments with ARP mRNA, GAPDH amplification plots were grouped together.

cells, a cell line derived from a human breast adenocarcinoma. Total RNA from given numbers of MCF-7 cells was analyzed against a background of total RNA from 10^4 cultured BHK cells (non-primate BHK cells do not express human BC200 RNA).[2]

The qRT-PCR amplification plot shows that the BC200 RNA approach can detect as few as three MCF-7 cells and, additionally, that it can detect small changes in the number of such cells (figure 4A,B). BC200 RNA is thus a highly sensitive indicator of invasive breast cancer cells in blood. Conversely, the mRNA encoding the housekeeping protein ARP produced amplification plots that clustered together (figure 4A,B), that is, did not reflect the number of MCF-7 cells used. This result was expected considering that the gene is constitutively expressed irrespective of cell type.

Analogous limiting dilution experiments were performed in which RNA from MCF-7 breast cancer cells was analyzed against a background of RNA from whole blood of healthy subjects. Using both ARP and GAPDH mRNAs as internal qRT-PCR standards, we were able to detect BC200 RNA from three MCF-7 cells (figure 4C,D). BC200 RNA levels in samples containing three MCF-7 cell equivalents were significantly elevated, compared with samples containing 0 MCF-7 cell equivalents (Student's t-test, $p=0.0325$). The strength of the qRT-PCR BC200 signal again increased proportionally with the number of MCF-7 cell equivalents (figure 4C,D). The amplification signal in 0 MCF-7 cell samples is consistent with the baseline expression of BC200 RNA observed in blood from healthy subjects (see also figure 1A,B).

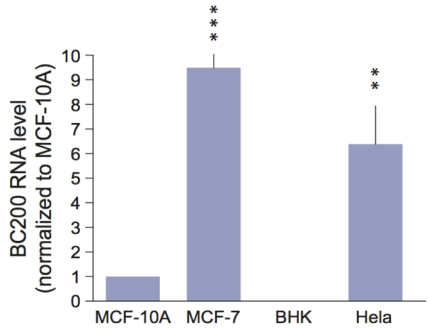

Figure 5 Expression of BC200 RNA in tumor cell lines. Expression levels in MCF-10A cells, MCF-7 cells, BHK cells, and HeLa cells were established by qRT-PCR. BC200 RNA expression was significantly elevated in MCF-7 cells and HeLa cells, in comparison with MCF-10A cells (the latter normalized to 1). Quantitative analysis: one-way ANOVA, p=0.0003; Dunnett's post hoc analysis, comparison of MCF-7 cells (p<0.001) and of HeLa cells (p<0.01) with MCF-10A cells. BHK cells were used as a negative control as expression of BC200 RNA is specific to primates.[2]

Expression of BC200 RNA in cancer cell lines

The above results with MCF-7 cells raise the question whether expression of BC200 RNA correlates with tumor cell invasiveness. To address this question, we established expression levels in MCF-7 cells and HeLa cells in comparison with levels in MCF-10A cells, a cell line derived from non-tumorigenic breast epithelial cells. BHK cells served as a negative control. Results from these experiments (figure 5) indicate that MCF-7 cells and HeLa cells express significantly more BC200 RNA than MCF-10A cells. Neither HeLa cells nor BHK cells are breast cancer cell lines. The HeLa cell result is therefore in confirmation of earlier data indicating that BC200 RNA is expressed in primary cervical cancer cells, although at moderate levels.[8]

HeLa cells are considered highly invasive.[23] MCF-7 cells, in contrast, are normally poorly invasive; however, invasiveness and metastatic activity can be induced, for example, by VEGF-C expression.[24] Expression of BC200 RNA in these two cell types is thus reminiscent of expression in invasive breast cancer and in ductal carcinomas in situ (DCIS). BC200 RNAs is expressed at high levels (similar to levels in invasive ductal carcinomas) in high-grade comedo DCIS but only at background levels in non-high-grade DCIS.[9] These results were interpreted to indicate that expression of BC200 RNA is associated with invasive carcinomas and potentially invasive ('preinvasive') carcinomas in situ. The data presented in figure 5 lend further support to this notion.

DISCUSSION

The significance of regulatory RNAs in eukaryotic cellular form and function is increasingly being recognized.[1 2] However, the role of such RNAs in tumorigenesis and tumor development, and their potential use as clinical tools, remains poorly understood and underexplored.[10] We have previously reported that human BC200 RNA is expressed at high levels in invasive breast cancer cells.[9] We now show that in patients with invasive breast cancer, BC200 RNA can be detected in peripheral blood with high specificity and sensitivity. The data illustrate the potential of BC200 RNA detection in blood as a minimally invasive molecular indicator of breast malignancies.

We have developed qRT-PCR methodology to discriminate BC200 RNA blood levels in patients with invasive breast cancer from those in healthy subjects and from those in patients with a past diagnosis of breast cancer but no evidence of recurrent disease. Not included in the present work were patients with benign, non-invasive breast tumors such as fibroadenomas as tissue expression levels of BC200 RNA in such tumors have previously been shown not to be significantly different from those in healthy breast tissue.[9] The difference in blood levels of BC200 RNA between untreated invasive disease subjects and healthy subjects is highly significant (p=0.001), and ROC analysis confirmed high specificity and sensitivity of the technique (AUC=0.89). Mammography has a median sensitivity of 79%, ranging from 74% to 85%.[25 26] A BC200 RNA blood test would therefore be a valuable clinical complement to mammography in screening for breast cancer. Of all malignancies examined, only adenocarcinomas of the breast, mucoepidermoid carcinomas of the parotid, and melanomas were found to express robust levels of BC200 RNA.[8] Thus, a screening result of elevated BC200 RNA blood levels would be an indication of the presence of one of these malignancies (which can easily be differentiated).

In patients with breast cancer who have undergone treatment (ie, resection and adjuvant therapy), average BC200 RNA blood levels were not significantly different from levels in healthy subjects, indicating that treatment has resulted in a significant reduction of BC200-expressing cells. This result is consistent with previous observations that treatment can reduce CTC numbers in blood.[22] However, BC200 RNA blood levels in postsurgery primary disease breast cancer patients were also not significantly different from those in presurgery patients, and normality analysis revealed that some patients in the postsurgery group exhibited elevated BC200 RNA levels even after treatment. We hypothesize that treatment efficacy may have been suboptimal in such cases and that, as a consequence, tumor cells will continue to be present in the circulation. Such information may be valuable for individual breast cancer treatment decisions and outcome predictions. CTCs derived from patients with breast cancer have been advanced as predictors of recurrent or metastatic disease.[27–29] We propose that high residual BC200 RNA blood levels after treatment can serve as an index of high relapse potential.

Tumor biomarker development has often been beset with difficulties.[30] Even prostate-specific antigen (PSA), widely used for prostate cancer screening, has been of debatable clinical use because of its rather poor sensitivity and specificity.[31] We suggest that one of the underlying reasons for the paucity of suitable molecular tumor markers is the limited stability and/or availability of tumor-relevant mRNAs or proteins. This contrasts with the high stability and abundance of a regulatory RNA such as BC200 RNA, molecular features that result in superior reliability and consistency of detection. Our work demonstrates the potential clinical use of BC200 RNA as a minimally invasive and highly discriminating molecular indicator of invasive breast malignancies.

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Contributors AI, GAS, and HT designed the overall experimental approach. Experiments were performed by AI, EQC, and RLC. Subject recruitment was overseen by EQC, RLC, and GAS; they also reviewed patients' records and oncological assessments. LA contributed to data presentation and manuscript preparation.

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