Molecular detection of disseminated breast cancer cells in the bone marrow of early breast cancer patients using quantitative RT PCR for CEA

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Received September 9, 2007

Carcinoembryonic antigen (CEA) is widely used as a serum tumor marker in various types of cancer. Several systems for the CEA-RT-PCR approach have been reported to date. In this study, we have evaluated the quantitative CEA-RT-PCR as a diagnostic tool for detection of isolated tumor cells in bone marrow of early breast cancer patients prior to the administration of any adjuvant systemic therapy.

We obtained bone marrow aspirates of 70 patients with stage I (37%), II (60%), and III (3%) breast cancer who underwent either immediate complete resection of the tumor or neoadjuvant therapy with subsequent curative surgery. mRNA was isolated using QIAamp RNA blood mini kit (Qiagen ®). Subsequently quantitative RT-PCR for the expression of CEA has been performed.

CEA transcripts were detected in samples from 29 (41%) out of 70 patients. With a median follow-up of 22 months we observed 8 disease free survival (DFS) events including 4 systemic recurrences, 1 ductal in-situ carcinoma (DCIS), 1 local recurrence, and 2 deaths without tumour. Four DFS events (2 systemic recurrences, 2 deaths without tumor) occurred in patients with CEA transcripts in the bone marrow and 4 (2 systemic recurrences, 1 DCIS, 1 locoregional recurrence) in patients without CEA in the bone marrow. There was a trend to shorter DFS in the group with CEA in the bone marrow (p=0.05548). Overall survival was not assessed because only 2 deaths (both in patients without tumor) have been reported to date.

Quantitative RT-PCR assay for CEA may be a useful tool for detection of occult breast cancer cells in the bone marrow. Clinical and prognostic relevance of minimal residual disease using this technique remains unproven. Our results should be interpreted with caution with regard to 2 deaths in CEA positive group with no relationship to disease recurrence.

Key words: Breast cancer, Bone marrow, CEA, Occult tumor cells, Minimal residual disease

Early systemic spread of breast cancer cells is considered to originate metastatic foci in distant organs, yet usually missed by conventional tumor staging [1]. Detection of occult tumor cells in the bone marrow of patients with early breast cancer may have prognostic significance and may contribute to development of new therapeutic strategies [2]. Various methods have been applied including immunocytochemical staining [3, 4] and the reverse transcriptase-polymerase chain reaction (RT-PCR) [5, 6, 7]. The RT-PCR method has been suggested as a procedure giving sensitivity 10- to 100- times higher than routine immunocytochemical methods [6]. However, RT-PCR in this context has proven controversial, because the specificity with which malignant cells can be detected depends on the number of amplification cycles and the design of the primers. Majority of genes encoding tumor-associated molecules is not limited to tumor cells, but is also found in certain normal tissues [8].

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Table 1. Patients' characteristics (n=70)

Characteristics	Patients	
	No.	%
Age		
Median 52		
Range 28-76		
Premenopausal	27	39
Postmenopausal	43	61
Stage		
I	26	37
IIA	19	27
IIB	23	33
IIIB	2	3
Node positive disease	35	50
Hormone receptors positive	62	89
Histology		
Invasive ductal carcinoma	53	76
Invasive lobular carcinoma	14	20
Mixed ductal/lobular carcinoma	3	4
Chemotherapy		
Neoadjuvant (F)AC*	4	6
antracylines-taxanes	3	4
letrozole	1	1
Adjuvant		
(F)AC/(F)EC *	37	53
CMF**	5	7
antracylines-taxanes	9	13
Tamoxifen	52	74
Anastrozole	2	3

* (F)AC/(F)EC: (fluorouracil), doxorubicin, cyclophosphamide/(fluorouracil), epirubicin, cyclophosphamide

**CMF: cyclophosphamide, methotrexate, fluorouracil

Carcinoembryonic antigen (CEA) was originally described as a glycoprotein molecule with an oncofetal expression pattern. It is widely used as a tumor marker of a variety of carcinomas, especially colon and breast cancer [9, 10]. Several systems for the CEA-RT-PCR approach have been reported to date. Gerhard et al. [6] have established a sensitive assay for the specific detection of CEA-expressing tumor cells in the bone marrow of patients with colorectal cancer and CEA-positive carcinomas. Their study has shown that the amplification of CEA-specific mRNA will allow specific and sensitive detection of single CEA- expressing tumor cells in 62 samples of the bone marrow and peripheral blood cells. Recently, Berois et al. [11] presented a study with 46 patients. CEA expression in the bone marrow was found in 17% of patients. Prognostic impact was not determined. Mitas et al. [12] have used quantitative CEA-RT-PCR for detection of occult tumor cells in axillary lymph nodes of breast cancer patients.

In this study, we have evaluated the quantitative CEA-RT-PCR as diagnostic tool for detection of isolated tumor cells in bone marrow of early breast cancer patients prior to the administration of any adjuvant systemic therapy.

Patients and methods

Patient and control population. Samples of bone marrow from sternum or iliac crest were obtained from patients with primary breast cancer treated in the Department of Oncology First Faculty of Medicine, Prague, Czech Republic. All patients enrolled were either post primary surgery with no evidence of disease or prior to a neoadjuvant therapy in case of locally advanced disease. Patients were enrolled between March 2001 and June 2004. Selection criteria included presentation with primary breast cancer stage I, II, and III according to AJCC [13], completion of an appropriate curative surgical procedure, or planned neoadjuvant therapy with curative intent. A total of 70 patients who satisfied these criteria were chosen: 26 (37 %) with stage I, 19 (27 %) with stage IIA, 23 (33 %) with stage IIB, and 2 (3 %) with stage IIIB. Detailed patients' characteristics are listed in Table 1. All patients received adjuvant therapy according to either St. Gallen consensus from 2001 [14] or from 2003 [15]. Adjuvant chemotherapy with antracycline, non-antracycline, or antracycline-taxane based regimen was delivered to 51(73 %) patients. Among 62 (89 %) patients with expression of hormonal receptors, 54 (77%) patients received appropriate adjuvant hormonal treatment with either tamoxifen or anastrozole. Eight receptor positive patients did not receive adjuvant hormonal therapy because they had not finished adjuvant chemotherapy or radiotherapy at the time of analysis. Nine premenopausal patients with persistent ovarian function underwent ovarian ablation either with goserelin or surgery in addition to hormonal therapy. Patients were treated with breast radiation when breast-conserving surgery had been performed. Patients with more than 3 positive axillary lymph nodes underwent axillary radiation. All individuals were observed for disease recurrence and death. Reference values for the basal expression of CEA in hematopoietic tissue were obtained from blood of healthy volunteers.

The study was conducted in accordance with the Declaration of Helsinki and was approved by the institutional Ethical Review Board. All patients or volunteers signed a written informed consent document.

Bone marrow/blood aspiration. To avoid epithelial contamination of bone marrow samples, the skin was incised before the aspirates were taken to minimize the risk of epithelial contamination. Between 0.5ml and 7.5ml (median 1.5ml) of bone marrow was aspirated from sternum (49 patients) or posterior iliac crest (21 patients) using disposable 15-gauge (1.8 mm) bone marrow needles (Allegiance Healthcare Corporation, McGaw Park, IL, USA) into syringes primed with EDTA. Peripheral blood of healthy donors was sampled from cubital vein into EDTA containing tubes. The samples were immediately processed as described in this section below (see RNA isolation and reverse transcription). Bone marrow aspiration either from sternum or posterior iliac crest as well as blood samples yielded sufficient amount of RNA for subsequent procedures.

RNA isolation and reverse transcription. Total RNA was extracted from bone marrow aspirates or blood using the commercial kit (QIAamp RNA Blood Kit, Qiagen, Valencia, USA) according to the manufacturer's protocol for fresh blood samples preparations. Concentration and purity of isolated RNA was assessed by a UV spectrometry. Three micrograms of total RNA were used for reverse transcription in a final volume of 30 µl. RNA solution was pre-incubated with 0.3 µg of random primers (Promega, Madison, WI) at 70 °C for 5 minutes and immediately put on ice afterwards. Then 6 µl of 5× reverse transcriptase buffer RevertAid (Fermentas, Vilnius, Lithuania), 3 µl of 10 mM deoxyribonucleotide triphosphates (dNTPs) and 0.75 µl of ribonuclease inhibitor RNAsin (40 U/µl) (Promega, Madison, WI) were added and left for 5 minutes at room temperature. In the final step, 150 U of Moloney murine leukaemia virus reverse transcriptase RevertAid (Fermentas, Vilnius, Lithuania) was added to each tube and samples were incubated at 42 °C for 60 minutes. Finally, reverse transcriptase was heat inactivated at 95 °C for 5 minutes.

Quantitative RT-PCR. Carcinoembryonic antigen was involved as a marker of minimal residual disease using modification of previously published method based on quantitative real-time PCR [16]. Primers were selected using PrimerPremier3 software and NCBI Reference sequences. Specific primers from two different exons and probes were selected (Generi-Biotech, Hradec Kralove, Czech Republic) to span introns to reduce amplification of contaminating genomic DNA. Following primers and probe were used: CEA3 5'–TAA GTG TTG ACC ACA GCG ACC C-3', CEA4 5'–GTT CCC ATC AAT CAG CCA AGA A-3', CEA probe 5'–ATG TCC TCT ATG GCC CAG ACG ACC C-3'-BHQ1-HEX, the length of the PCR product was 167 bp. RQ RT-PCR reaction was performed in 25 µl reaction volume consisting of 1 U of HotStart Taq Polymerase, 3 mM $MgCl_2$, 10× PCR buffer (AB Gene, Epsom, UK), 200 µM dNTPs (Promega, Madison, WI, USA), 100 ng cDNA, 300 nM CEA3, 600 nM CEA4 and 200 nM CEA probe. The optimized thermal profile for amplification was initiated with 15 minutes Taq polymerase activation at 96 °C, followed by 50 cycles at 95 °C for 15 s and 65 °C for 15 s. Standards were manufactured by cloning of longer DNA fragments (513 bp) prepared by PCR amplification using primers CEA3outer (5'–ACA GTC TAT GCA GAG CCA CCC AAA-3') and CEA4outer (5' – GCT GTG GCC ACT GGC TGA GT – 3'). Amplified DNA was cloned applying Topo TA Cloning Kit into pCR 2.1-Topo plasmid (Invitrogen, Carlsbad, California). Concentration and purity of isolated plasmid DNA was assessed by UV absorbance method. Calculation of CEA absolute copy number in plasmid DNA was based on known plasmid molecular weight and Avogadro number (6.0236x 10^{23} molecules.mol^{-1}). Gene expression was quantified based on calibration curves using appropriately diluted cloned gene specific standards ranging between 10^2 – 10^9 copies per reaction (Figure 1). Each sample was analyzed in duplicate. Positive and negative controls were also incorporated into each experiment in order to evaluate reproducibility and eventual sample contamination during the PCR procedure. The final results were presented as absolute copy numbers of CEA mRNA per 1 µg of total RNA.

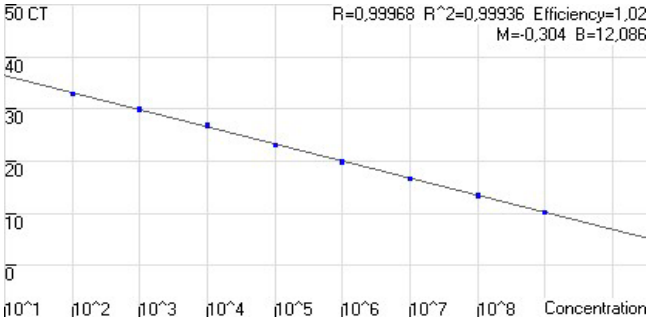


Figure 1. Typical example of calibration curve used for quantitative analysis of CEA gene expression. Ten-fold serial dilutions of standard plasmid DNA containing cloned fragment of CEA gene were made in 1mM Tris buffer. Individual dilutions corresponded to 10^2 – 10^9 copies per reaction. Each sample was analyzed in duplicate and only mean values were plotted.

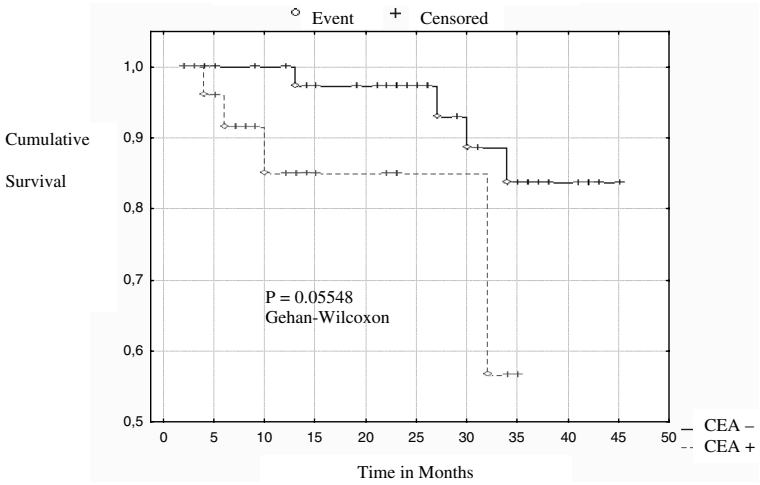
Statistical analysis

Statistical analysis was performed using software Statistica version 6 (StatSoft © 2003). We assessed correlation between CEA expression and other prognostic variables such as tumour size, infiltration of axillary lymph nodes, number of nodes involved, hormone receptors expression, histological type, and tumor grade. Furthermore, we performed univariate analysis of CEA in bone marrow and disease free survival (DFS), distant metastasis free survival (DMFS) and relapse free survival (RFS). DFS was defined as the time from entering the study to distant metastasis, locoregional recurrence, contralateral breast cancer, ductal carcinoma in-situ (DCIS), or death without tumor. DMFS was defined as the time from entering the study to distant metastasis. RFS was defined as the time from entering the study to distant metastasis, locoregional recurrence, contralateral breast cancer, or ductal carcinoma in-situ (DCIS).

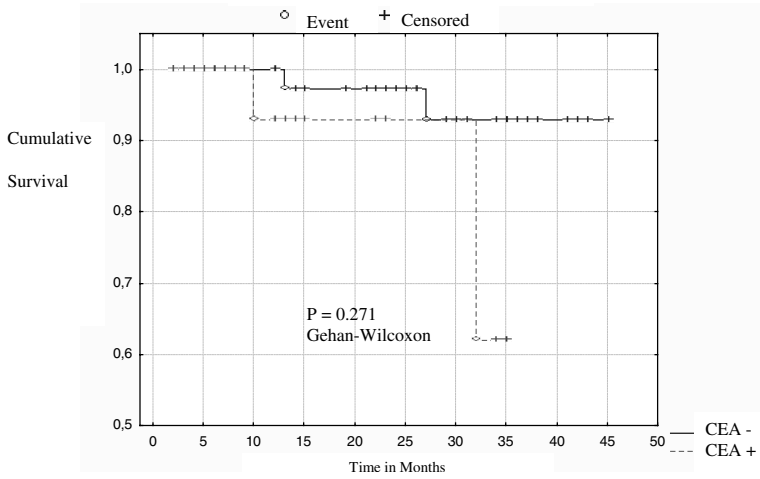
Results

We have successfully introduced sensitive and robust real time PCR method for quantitative determination of CEA expression in bone marrow and blood of breast cancer patients and/or healthy volunteers as a marker of minimal residual disease. The results of gene expression were normalized rather to the amount of total cellular RNA than to housekeeping gene(s) in order to avoid inter- and intra- individual variability. Since the background CEA mRNA expression was also found in non-malignant hematopoietic tissues, we have determined normal reference values analyzing illegitimate CEA transcription in the blood of 58 adult healthy blood donors.

A) DFS according to Kaplan Maier



B) DMFS according to Kaplan Maier



C) RFS according to Kaplan Maier

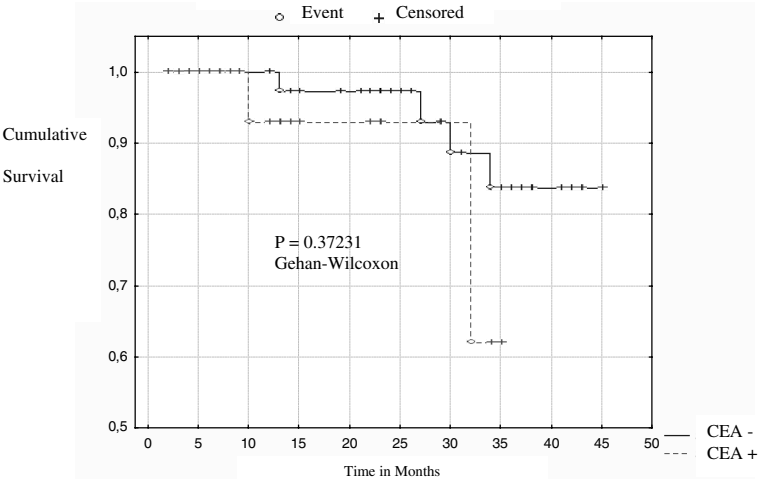


Figure 2

DFS, Disease free survival; DMFS, Distant metastasis free survival; RFS, Relapse free survival.

Low levels of CEA mRNA expression were detected in all tested control samples. Mean value of CEA expression was 83±65,9 gene copies per µg of RNA. Cut-off value for minimal residual disease positivity in hematopoietic compartments was determined as 250 copies/µg of RNA (mean value plus 2 SD). None of the healthy donors had CEA expression above the cut-off value.

Bone marrow aspirates from 70 patients were collected prior to administration of any systemic therapy. Satisfactory amount of mRNA was obtained in all 70 patients enrolled. CEA mRNA transcripts were detectable in bone marrow of 29 (41%) patients (Table 2). The majority of these patients had stage II disease: 9 stage IIA and 9 stage IIB. Ten patients had stage I and only one had stage IIIB. Slightly more than half of them (15/52%) had N+ disease (involvement of axillary lymph nodes). When compared to other prognostic features there was no correlation with tumor grade, hormone receptor status, and histological type (respectively: r = -0.22; r = 0; r = 0.06; p>0.05 in all cases).

The median follow-up period at the time of analysis was 22 months (range, 2 to 45 months). Eight patients (9%) had experienced events recorded for DFS. 4 patients developed distant metastases, 1 locoregional recurrence, 1 DCIS, and 2 died without tumor (pulmonary embolism with no recurrence on the autopsy and 1 car accident). Among patients with CEA transcripts in the bone marrow we observed 2 systemic recurrences and 2 deaths without tumor. In patients without CEA in the bone marrow 2 systemic recurrences have been reported, 1 DCIS and 1 locoregional relapse. There was a trend to shorter DFS in the group with CEA in the bone marrow, however it was just above the level of statistical significance (p=0.05548, Gehan-Wilcoxon). There was no difference between both groups as regards to DMFS and RFS (respectively: p=0.271, p=0.37231). DFS, DMFS, and RFS curves are displayed in Figure 2A-C. Since only 2 deaths (both not related to tumor) have been reported to date, overall survival has not been evaluated.

Discussion

The detection of occult tumor cells in the bone marrow of patients with early stages breast cancer might contribute to more accurate staging predicting disease relapse and shorter overall survival [17]. Several markers including cytokeratins, mammaglobin, and maspin have been tested to date for RT PCR detection of minimal residual disease in patients with various carcinomas including breast cancer [18, 19, 20]. CEA was investigated as a marker of occult tu-

mor cells in lymph nodes, samples of peripheral blood, and bone marrow aspirates. Min et al. [21] has shown excellent diagnostic utility of RT PCR CEA in detection of occult tumor cells in sentinel lymph node biopsies. Mitas et al. [12] achieved similar results using quantitative RT PCR technique. In opposition others concluded that CEA had little diagnostic value in the same setting [22]. Unfortunately, all of these studies included small number of patients (from 17 to 22), thus no conclusion could have been drawn. Similar conflicting results were obtained when using peripheral blood [23]. Based on the review of literature, CEA mRNA transcripts in the bone marrow were found from 17.4% to 67 % of patients. Gerhard et al. [6] detected CEA using RT PCR in 4 out of 6 breast cancer patients. Zhong et al. [24] detected CEA in 27.8% of patients in the largest series published to date (181 patients after breast surgery) using the similar technique. Berois et al. [11] reported the smallest proportion of CEA positive bone marrow samples (17.4%; 8 out of 46 patients). We diagnosed minimal residual disease in 41% (29 out of 70) early breast cancer patients using the quantitative RT PCR technique.

In previously published studies the presence of minimal residual disease in the bone marrow using various immunohistochemical assays was identified as an independent negative prognostic factor for disease free and overall survival of early breast cancer patients [17]. Moreover, just recently published meta-analysis confirmed these findings [25]. CEA as a marker of occult tumor cells in peripheral blood showed negative prognostic value in patients with colorectal and non-small cell lung cancer [26, 27]. In breast cancer, Jotsuka et al. [28] published results from series of 101 consecutive early breast cancer patients. They identified that CEA mRNA transcripts detected in peripheral blood adversely affected patients' prognosis. Stathopoulou et al. [29] evaluated CEA in blood samples from patients with breast, colorectal or hematological malignancies without reporting survival data. No larger study concerning prognostic value of CEA in the bone marrow has been published thus far. Overall in our study in the median follow up of 22 month we observed 8 DFS events, 4 in patients with CEA transcripts in the bone marrow. However 2 events in the group with CEA in the bone marrow were not tumor related. There was a trend to shorter DFS in the group with CEA in the bone marrow, which is, however, just above statistical significance (p=0.05548). This finding was not confirmed by means of DMFS and RFS. DFS data should be interpreted with extreme caution since it may be influenced by 2 tumor unrelated death in the subgroup with proven CEA transcripts in the bone marrow. No correlation with other clinically used prognostic variables has been found. Our study showed that CEA might be used as a marker of minimal residual disease in the bone marrow of patients with early stages breast cancer, however its specificity should be further evaluated on bone marrow samples of healthy volunteers to exclude the possibility of illegitimate transcripts in normal bone marrow. The prognostic significance of CEA remains to be further investigated.

Table 2. Distribution of CEA in the bone marrow according to the stage and axillary lymph nodes involvement.

	CEA + /%	CEA- /%
All patiens (N=70)	29 out of 70 / 41	41 out of 70 / 59
Stage I	10 out of 29 / 35	16 out of 41 / 39
Stage IIA	9 out of 29 / 31	10 out of 29 / 25
Stage IIB	9out of 29 / 31	14 out of 29 / 34
Stage IIIB	1out of 29 / 3	1 out of 29 / 2
Axillary lymph nodes involvement	15 out of 29 /52	20 out of 29 / 49

No correlation observed between CEA in the bone marrow and other prognostic variables.

The study was supported in parts by grant projects NR7804, MPO 1H-PK45 and the research concepts MSM0021620808 and MSM6198959216.

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