Detection of minimal residual disease in patients with early breast cancer

(Thesis-Short Version)

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Contents

The list of abbreviations

cDNA	Complementary DNA
CEA	Carcinoembryonic antigen
CK	Cytokeratin
DCIS	Ductal carcinoma in-situ
DFS	Disease free survival – time to recurrence, tumor unrelated death, contra lateral or secondary tumor
DMFS	Distant metastasis free survival- time to distant metastases
DNA	Deoxyribonucleic acid
EDTA	Ethylenedinitrilotetraacetic acid
EGFR	Epidermal growth factor receptor
EpCAM	Epithelial cells adhesive molecule
HER-2/neu	Human epidermal growth factor receptor 2
HER3	Human epidermal growth factor receptor 3
HER4	Human epidermal growth factor receptor 4
mRNA	Messenger RNA
mTOR	Mammalian target of Rapamycin
N0	Node negative
N+	Node positive
OS	Overall survival
p53	Tumor suppression protein 53
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RR	Relative risk
RFS	Relapse free survival – time to local or distant recurrence or carcinoma in-situ
RT-PCR	Reverse transcriptase polymerase chain reaction
VEGF	Vascular endothelial growth factor

1. Introduction

According to literature approximately half of patients with a primary operable breast cancer eventually develop a disease recurrence within 5 years (1). In patients without infiltration of axillary lymph nodes recurrence occurs only in 25% of patients (2). There is an increased demand for identification of new prognostic factors which would distinguish patients already cured with surgery from those who need an additional treatment.

Occult metastases which are present at the time of diagnosis lead to the overt metastatic disease and the shorter survival (3). Unfortunately, these occult metastatic lesions are far bellow detection limits of routinely used techniques (4).

An adjuvant treatment is given to reduce the risk of recurrence and prolong survival (5-8). Possible side effects are significant limitations of the treatment. Postoperative therapy is currently indicated based and published results of large international randomized trials, but there is no available method to prove the efficacy in the every individual patient (5). Recently developed mathematical models help us to determine the risk but they again work mainly with data obtained from randomized trials (9). Minimal residual disease might be helpful for identification of patients in the increased risk of relapse and possibly in monitoring of response to the adjuvant treatment.

Minimal residual disease could be monitored in bone marrow, peripheral blood and lymph nodes (10).

2. Current advances

2.1. Immunocytochemical detection and the prognostic impact of minimal residual disease in bone marrow

Antibodies against cytokeratins and epithelial mucins are most commonly used in detection of minimal residual disease (11-15). Up to now there have been published 27 studies with 6228 patients investigating minimal residual disease in bone marrow in international medical journals or presented at major international conferences (11-38). Results are not easily comparable due to large variability in methods and sample size. It is not a surprise that the first published meta-analysis with 20 studies and 2494 patients was negative in terms of prognostic significance (39). Nevertheless, 16 out of previously mentioned 27 clinical studies have showed the shorter disease free or the overall survival associated with minimal residual

disease. Moreover, in 11 studies (3772 patients) these results were supported by the multivariate analysis (10). The most recent meta-analysis published recently in the New England Journal of Medicine by Braun et al. (40) evaluated 9 large studies with comparable methodologies. In total, 4703 patients were enrolled. The prevalence of minimal residual disease was slightly above 30%. It has correlated with other prognostic factors such as a tumor size, tumor grading, infiltration of axillary lymph nodes, hormone receptor negativity. Patients with minimal residual disease in bone marrow had significantly worse 10 year survival (RR 2.26, p=0.007).

2.2. Immunocytochemical detection and the prognostic impact of minimal residual disease in peripheral blood

Circulated tumor cells were firstly described in 1869 by Ashworth, who identified them in blood of deceased (41). Engel (42) published in 1955 a systematic review proving the presence of circulated tumor cells in patients with advanced cancers. Due to technical limitations in 60's circulated tumor cells were detected in 1% of patients only (43). The major advance has been brought up with introduction of immunocytochemical methods. Redding (44) in the year 1983 published remarkably higher detection of circulated cancer cells with immunocytochemistry in comparison with conventional techniques. Searching currently available literature there have been only 8 systematic studies identified. The most of them are small not focusing on a prognostic impact.

2.3. Immunohistochemical detection and the prognostic impact of minimal residual disease in the lymph nodes

The infiltration of axillary lymph nodes is the most important prognostic factor in an early stage breast cancer (45). Using immunohistochemistry cancer cells are identified in 14-30% (16% in average) initially negative samples (46,47). However the prognostic significance of occult metastatic cells detected by immunohistochemistry remains unproven (48-50). Because these studies were relatively small it is debatable if they had power to prove significant difference in terms of disease free survival (DFS) or overall survival (OS) (51). These drawbacks did not have a study carried out in Ludwig's Institute in Munich in 921 node negative patients enrolled (52). Second opinion reading of hematoxillin eosin slides showed

metastasis in 9% patients. These patients had worse prognosis in terms of DFS (p=0,003) and OS (p=0,002). Similar result brought a study published by Neville and Mascarel (53,54). Nevertheless, the subsequent multivariate analysis failed to prove prognostic significance of occult tumor cells. Immunohistochemistry was an important milestone. The prognostic impact was shown in several such studies (55-58). But overall data are still quite conflicting and patients with occult tumor cells on immunohistochemistry only have to be still classified as N0 (node negative) (45).

2.4. Detection using flow cytometry

Flow cytometry provides conflicting data despite declared sensitivity about $1:10^7$ (29,59-62). Number of positive trials is similar to those which are negative (63-68).

2.5. Molecular detection of minimal residual disease

The most common molecular method is a reverse transcriptase polymerase chain reaction (RT-PCR). The specificity depends on a number of amplification cycles and design of primers. Moreover, commonly used cytokeratin primers are not tumor specific markers of breast cancer. The declared sensitivity of RT-PCR leis between $1:10^6$ and $1:10^8$ (69).

The specificity of many RT-PCR-based assays for the detection of cancer cells is a matter of discussion. This poses enormous problems for interpreting the obtained results and drawing conclusions. False positive results could be produced owing to possible contamination of samples with epithelial cells. It explains positive results in some of healthy volunteers (70). The result of RT-PCR may be influenced due to contamination with genomic deoxyribonucleic acid (DNA). Another confounding factor could be a contamination of reaction with the polymerase chain reaction (PCR) product from previous reactions in the same laboratory. Studies with RT-PCR are also frequently criticized because of possible illegitimate gene transcription (71-74).

Datta et al. detected transcripts of cytokeratin (CK) 19 in 26% cases of 34 enrolled patients (75). Minimal residual disease in bone marrow was associated with shorter DFS in the univariate analysis. Several other studies showed similar results (75-80). On the other hand a few studies did not confirm or investigate prognostic significance (75-83,91). Overall 18 studies with 1193 enrolled patients have been identified. Transcripts indicating dissemination of tumor cells were found in bone marrow of 9-81% of patients. The prognostic

significance in terms of shorter DFS was confirmed in 6 studies in the univariate analysis and in 2 in the multivariate analysis. In contrary 8 studies did not show any prognostic significance and 5 studies did not address this issue.

Minimal residual disease was also investigated in peripheral blood. These studies were predominantly small with 23 to 206 patients enrolled (81,82,89-103). Most of them used cytokeratins as markers. Circulated tumor cells were detected in 3-60% enrolled patients. The prognostic significance has been rarely investigated (82,92,93,97,102).

An attractive indication especially in the context of sentinel node biopsy would be examination of axillary lymph nodes (104,105). None of these trials showed any prognostic significance, however they were usually not designed to do so. Transcripts of minimal residual disease were found in 20-38% negative nodes on routine histology (106-108). These finding at the moment does not justify upstaging from N0 to N+ (node positive) group (109).

2.6. Caveats in detection of minimal residual disease

Immunocytochemical methods using monoclonal and/or polyclonal antibodies are capable to discriminate cells of different origin (e.g. epithelial cancer cells from hematopoiteic or stromal cells of bone marrow or lymph nodes). Majority of antibodies is targeted against specific epithelial antigens. None of these antibodies is specific for tumor and they have possible cross-reactivity with tumor cells and normal epithelial cells. Results might be confounding due to contamination of the sample with epithelial cells (108). The specificity of antibodies is also a key issue. Some epithelial mucins and other membrane antigens could be expressed on hematopoietic precursor cells like erythroblasts (108,110,111).

The PCR reaction is extremely sensitive which may lead to false positive results (112). It may be caused for instance by illegitimate transcription. Tumor samples could be also contaminated the same way as mentioned before (113). Another limiting factor of PCR is a relatively short half life of mRNA (messenger RNA) which requires immediate processing of samples after collection.

Cytokeratins are the most frequently used markers (CK 19 and CK 20). As stated before cytokeratins are common epithelial markers not specific for breast epithelium which may be responsible for the lower specificity of molecular methods in this indication (10,114). In contrary mammaglobin is a new breast specific marker which is currently being investigated (115). It belongs to the family of uteroglobine genes and its function in human

organism remains unclear. Mammaglobin overexpression is specific for normal breast and neoplastic tissue (116). Mammaglobin is expressed in 80-90% of primary tumor samples with 100% specificity (117). Mammaglobin is being investigated mainly in the context of RT-PCR detection. We have only limited experience with immunocytochemical detection so far (117). Among other possible markers carcinoembryonic antigen (CEA) is tumor specific but not necessarily for breast cancer. CEA expression could be detected in significant proportion of colorectal cancers (118).

2.7. Minimal residual disease in the monitoring of efficacy of adjuvant treatment

The postoperative adjuvant treatment is currently indicated on the grounds of published results of randomized clinical trials. The efficacy could not be predicted or validated in an individual patient. It is not obvious whether occult tumor cells are real markers of hematogenous spread or rather dormant cells with a low proliferative activity and low malignant potential (119). A low proliferative activity may also explain why currently used cytotoxic regimens fail to eradicate occult tumor cells. Braun et al. (120) conducted a trial in 59 high risk breast cancer patients. Occult tumor cells were investigated using immunocytochemistry in the beginning and after completion of the adjuvant chemotherapy (anthracycline or taxane containing regimens). Prevalence rates of minimal residual disease pre and post chemotherapy were similar however some patients initially positively tested for minimal residual disease have become negative and vice versa. Anyhow the presence of cytokeratin positive cells after completion of adjuvant treatment was a strong negative prognostic factor. Similarly inconclusive data were published from two high dose chemotherapy trials (121,122).

Because chemotherapy seems to be failing to eliminate occult tumor cells new biologic agents are explored. Ten patients with advanced breast cancer were treated with 1 dose of edrecolomab, a monoclonal antibody against epithelial adhesive molecule EpCAM expressed on breast cancer cells (123). All patients demonstrated improvement in bone marrow 5 - 7 days after administration. This approach has to be validated in larger clinical studies.

3. Study objectives

1. Implementation of methods for detection of minimal residual disease.
   a. Detection of CK19 expression using immuno(cyto)histochemistry in axillary lymph nodes and bone marrow.
   b. RT-PCR detection of mammaglobin A, B, and CK19 expression in bone marrow.
   c. Quantitative RT-PCR detection of CEA expression in bone marrow.
2. Detection of minimal residual disease using specific markers and techniques.
3. Assessment of the prognostic significance of minimal residual disease.
4. Correlations between minimal residual disease and other clinical and histopathological prognostic factors.

4. Methods

4.1. Patient's population

Patients with early or locally advanced breast cancer (stages I,II, and III) treated in years 2001-2005 in the Department of Oncology 1st Faculty of Medicine and General Teaching Hospital were offered to participate in the study if they were after radical surgery before any adjuvant treatment or prior commencement neoadjuvant therapy with curative intent (124). All patients received adjuvant therapy according to either St. Gallen consensus from 2001 (125) or from 2003 (126).

The study was conducted in accordance with the Declaration of Helsinki and was approved by the institutional Ethical Review Board. All patients signed a written informed consent document. In total 91 patients were enrolled.

4.2. Sample collection and processing

Samples of lymph nodes were available only for immunohistochemical analysis.

Bone marrow samples were aspirated from sternum or posterior iliac crest using disposable 15-gauge (1.8 mm) bone marrow needles (Allegiance Healthcare Corporation, McGaw Park, IL, USA) and syringes primed with EDTA (ethylenedinitrilotetraacetic acid). To avoid epithelial contamination of bone marrow samples, the skin was incised before the aspirates were taken to minimize the risk of epithelial contamination. The samples were processed immediately.

Subsequently all bone marrow aspirates or samples of peripheral blood were processed using gradient centrifugation on Percoll for further immunocytochemical analysis.

Samples designated for cell cultures were ressupended in culture media EMA on the culture plate for 48 hours. They were kept in thermoregulator at 37 $^{\circ}$C and 3.5% concentration of CO_2. Subsequently immunocytochemistry was carried out.

Samples appointed for RT-PCR were immediately submitted for RNA isolation. Total ribonucleic acid (RNA) was extracted from bone marrow and peripheral blood samples using a commercial kit (QIAamp RNA Blood Kit, Qiagen, Valencia, USA) according to

manufacturer's instruction. Quantity and purity of RNA was assessed on a spectrophotometer and on agarose gel.

The complementary DNA (cDNA) for nested RT-PCR was prepared using MMuLV Expand Reverse Transcriptase (Roche) and random hexanucleotids (Roche) according to manufacturer's instructions. Shortly, 2 μg RNA with 20 pmol of hexanucleotids were incubated for 10 minutes at 65°C. The reverse transcriptase reaction was carried out after addition 4 μl RT of buffer (Roche), 2 μl 100 mM DTT, 50U MMuLV Expand Reverse Transcriptase a 2 μl 10 mM dNTPs. Reaction mixture was incubated for 45 minutes at 42°C and transferred on ice.

The detection of a specific product (mammaglobin A, mammaglobin B, CK 19) was carried out using two-step PCR amplification. Single-step amplification of the transcript for β-globin was used as a marker of integrity of cDNA (or isolated RNA). PCR itself was performed under optimalised condition for annealing temperature, the number of cycles, the amount of templates, the concentration of $MgCl_2$ and primers. The specificity of PCR product was verified on sequenator (BigDye 3.1., ABI 310). The PCR reaction was carried out using a PTC200 Dyad PCR machine (MJR). PCR was carried out using specific primers for detection of mammaglobin A, B, and CK 19.

cDNA for quantitative RT-PCR was prepared using kit RevertAid (Fermentas, Burlington, Canada) on gradient cycler Thermal Cycler PTC 200 (MJ Research, Waltham, MA). Originally published primers for CEA detection (accession numbers NM_004363, NM_002354 and NM_002046 respectively) (127) were modified and designed to prevent amplification of similar genes or genomic DNA. The real-time RT-PCR was carried out using cycler Rotor-Gene 3000 (Corbett-Research, Sydney, Australia). Each sample was analysed in doublets. Both positive and negative controls were performed to verify sensitivity, specificity, reproducibility and possible contamination. Real-time RT-PCR reaction was evaluated on softwar Rotor-Gene 3000 version 6.0 (Corbett-Research, Sydney, Australia). Absolute quantification of a detected mareker was compared to the ammount RNA entering reverse transcription.

Imunocytochemical analysis of wet bone marrow cells and peripheral blood was performed using a sedimentation chamber. Some samples were cultured in-vitro for 48 hours. Cells stained with a mouse monoclonal antibody A53-B/A2 against CK 19 (Immunotech ®) were deemed to be neoplastic. Five microns slices were used for immunohistochemical analysis of lymph nodes. Afterwards two step standard reaction with antihuman mouse

monoclonal antibody against CK 19 with visualization using classical peroxidase-antiperoxidse technique.

4.3. Statistical analysis

Statistical analysis was performed on software Statistica version 6 (StatSoft © 2003). There were evaluated correlations between minimal residual disease and other prognostic factors. The univariate analysis of minimal residual disease and disease free survival was carried out.

5. Results

5.1. Patients populations

Ninety one patients who met inclusion criteria were prospectively investigated. Forty eight of them consented to have follow-up investigation after completion of the adjuvant treatment or after minimum of 6 months in case of neoadjuvant treatment. Remaining 43 patients did not provide their consent or were lost from follow up. Results were evaluated separately for different markers.

5.2. Minimal residual disease in bone marrow using nested RT PCR for CK19, mammaglobin A, and mammaglobin B

Seventy patients were prospectively investigated prior any adjuvant or neoadjuvant treatment. Twenty six of them underwent the follow up aspiration of bone marrow after adjuvant treatment (or after minimum of 6 months in case of hormone treatment). In 19 patients the follow up collection was planned and 25 patients did not consented with follow up studies. Breast cancer recurrence was detected in 2 patients. Unfortunately both rejected aspiration of bone marrow at that time. The representative RNA was isolated in 51 out of 70 samples taken before the treatment and in 24 out of 26 samples taken after the treatment. Only representative samples will be presented in results. Patients' characteristics are in table 1.

Table 1: Patients with representative RNA samples for RT PCR (N=56)

Variable		Patients	
		No.	%
Age			
Median	53		
Range	28-76		
Representative RNA in pre-treatment samples only		32	57
Representative RNA in post-treatment samples only		5	9
Representative RNA in both samples		19	34
Stage			
I		20	36
IIA		13	23
IIB		21	37
IIIB		1	2
Undetermined		1	2
N+ [*]		27	53
HR + [**]		47	84
HER2/neu +		22	40
Histology			
Invasive ductal carcinoma		49	88
Invasive lobular carcinoma		6	10
Undetermined		1	2
Chemotherapy			
Neoadjuvant (F)AC [***] or ET [****]		4	7
Adjuvant			
(F)AC [***]		28	50
CMF [*****]		4	7
anthracyclines-taxanes		8	14
Tamoxifen or different hormone treatment (AI) [******]		47	84

[*] N+: node positive

[**] HR+: hormone receptors positive

[***] (F)AC: (fluorouracil), doxorubicin, cyclophosphamide

[****] ET: epirubicin, docetaxel

[*****] CMF: cyclophosphamide, methotrexate, fluorouracil

[******] AI: aromatase inhibitors

5.2.1. Nested RT-PCR detection of mammaglobin A in bone marrow.

Seventy patients were investigated before commencement of adjuvant/neoadjuvant therapy. Mammaglobin A expression was investigated in 51 samples with sufficient amount of RNA. Mammaglobin A was detected in 6 (12%) out 51 patients. Mammaglobin A expression correlated with number of infiltrated axillary lymph nodes (r=0,29; p=0,045) and histopathologic grading (r=0,32; p=0,024). Other correlations were not statistically significant (stage, infiltration of axillary lymph nodes, histology subtype, hormone receptor expression and HER2/neu).

Mammaglobin A was identified in 2 (8%) out of 24 representative follow up samples. Both samples had been negative for mammaglobin before therapy started. Mammaglobin A expression correlated with low expression of hormone receptors (r= -0,46; p=0,03). Other correlations were statistically not significant.

PCR results are demonstrated on picture 1.

Picture 1: Mammaglobin A and B RT-PCR detection in bone marrow. Electrophoresis results after second PCR. Mammaglobin A transcripts are detected in sample 2 and 5.
M – DNA mark, A – nested PCR for mammaglobin A (219 bp), B – nested PCR for mammaglobin B (245 bp), neg.C. – negative control, pos.C – positive control (cDNA obtained from RNA from a breast cancer sample)
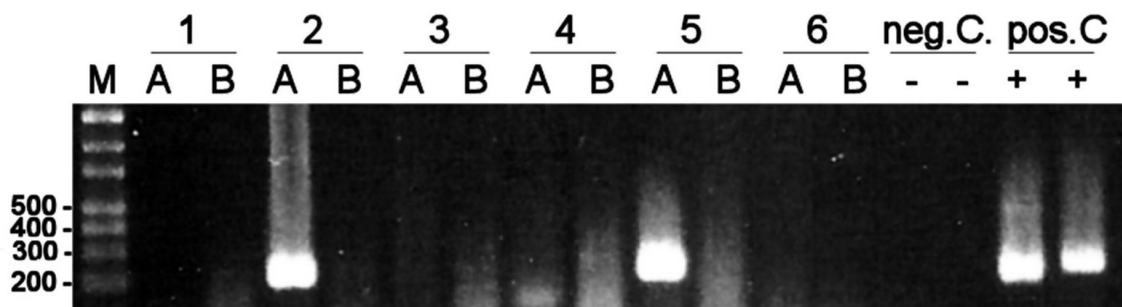
5.2.2. Nested RT-PCR detection of mammaglobin B in bone marrow

Mammaglobin B specific mRNA was identified in 2 (4%) out of 51 patients with representative RNA sample. One patient had also proven transcript of mammaglobin A. Mammaglobin B correlated with low expression of hormone receptors (r=-0,38; p = 0,007). Other correlations have not been identified (e.g. stage, infiltration and number of infiltrated axillary lymph nodes, histology subtype, histopathologic grading and expression of HER2/neu).

Mammaglobin B was detected in 2 (8%) out of 24 follow up samples. In one case it was persistent expression after therapy. This patient had also proven mammaglobin A transcripts in the follow up sample. When compared with other prognostic factors mammaglobin B expression correlated with low expression of hormone receptors (r= - 0,67; p = 0,0006). Other correlations were not statistically significant (stage, infiltration of axillary lymph nodes and their number, histology subtype, histopathologic grading and expression of HER2/neu).

5.2.3. Nested RT PCR detection of CK 19 in bone marrow

CK 19 transcripts were found in all 51 representative samples taken prior adjuvant/neoadjuvant treatment (picture 2) therefore it was not useful to pursue that investigation (see discussion).

Picture 2: RT-PCR detection of CK 19 in bone marrow. Electrophoresis after second PCR. CK 19 transcripts are detected in all samples.
M – DNA mark, 1-20 - sample numbers, NC. – negative control, PC – positive control (cDNA obtained from RNA from a breast cancer sample)

M 1   3   5   7   9   10   12   14   16   18   20PCNC
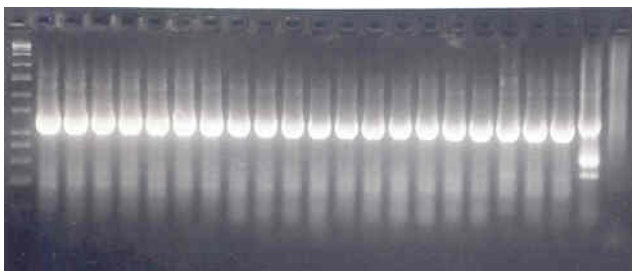
5.2.4. Mammaglobin A and B in bone marrow and DFS

5.2.4.1. Before chemotherapy

First of all it is important to stipulate that in the whole population of 51 patients only 2 recurrences were identified. It is the smallest possible amount for statistical analysis. Only 2 deaths were observed in study population. None of them was tumor related (pulmonary embolism, road traffic accident) thus evaluation of OS would not have any significance. Both recurrences were diagnosed in patients without mammaglobin A or B expression in bone marrow. Neither statistical differences for mammaglobin A (p=0.95) nor mammaglobin B (p=0.79) in terms of DFS were observed.

5.2.4.2. After chemotherapy

In the subgroup of 24 patients with good quality RNA investigated after completion of adjuvant treatment or at least after 6 months in case of hormone therapy. There was diagnosed only 1 recurrence in a patient with expression of mammaglobin A in bone marrow. Sample collected before treatment from the same patient was negative for both mammaglobin A and B. Statistical evaluation has not been done due to only one recurrence reported.

5.2.5. Quantitative CEA RT PCR detection of minimal residual disease in bone marrow

CEA expression was evaluated later. At that time 70 samples of representative mRNA were available. Only patients before adjuvant or neoadjuvant treatment have been investigated. Detailed patients' characteristics are in table 2. All patients were followed for disease recurrence or death.

Table 2: Patients population (N=70)

		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
N+[*]		35	50
HR +[**]		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
	anthracycline-taxane	3	4
	letrozole	1	1
	Adjuvant		
	(F)AC/(F)EC [***]	37	53
	CMF[****]	5	7
	anthracycline-taxane	9	13
Tamoxifen		52	74
Anastrozole		2	3

[*] N+: node positive

[**] HR+: hormone receptors positive

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

[****] CMF: cyclophosphamide, methotrexate, fluorouracil

5.3. Quantitative CEA RT-PCR detection in bone marrow

CEA mRNA transcripts were detected in bone marrow samples of 29 (41%) patients. Most of them were stage II (9 stage IIA, 9 stage IIB). More than half of patients had axillary lymph nodes infiltrated (15/52%). There were no significant correlations with other prognostic factors as follows: grading (r = -0.22), hormone receptor expression (r = 0), and histology subtype (r = 0.06).

Median follow-up at the time of analysis was 22 months (range: 2 to 45 months). Eight DFS events (disease recurrence, non cancer related death, contra-lateral breast cancer or secondary cancer) have been observed. Four patients experienced distant metastases, 1 loco-regional recurrence, 1 ductal carcinoma in-situ (DCIS) and 2 non cancer related deaths (pulmonary embolism, road traffic accident). In patients with CEA transcripts in the bone marrow 2 distant recurrences and 2 non cancer deaths have occurred. In patients without CEA in bone marrow 2 distant recurrences, 1 DCIS and 1 loco-regional recurrence were observed. There was a trend towards shorter DFS in patients with CEA in bone marrow. Nevertheless, it has been just above the level of statistical significance (p=0.05548, Gehan-Wilcoxon) (graph 1). There were no statistically significant differences between subgroups in terms of distant metastasis free survival (DMFS- time to distant metastasis) (p=0.271), and relapse free survival (RFS - time to local recurrence, distant recurrence, or DCIS) (p=0.37231). OS has not been evaluated because of small number of recurrences (both not cancer related).

Data regarding prognostic significance in terms of DFS should be interpreted with extreme caution because they are influenced by 2 non cancer related deaths in patients with CEA expression in bone marrow.

However quantitative CEA RT-PCR detection could be used for detection of minimal residual disease in the bone marrow of patients with early stages of breast cancer. Nevertheless, the specificity of this technique should be verified on bone marrow samples of healthy donors. Prognostic significance of CEA has to be determined.

Examples of real-time RT-PCR record are demonstrated on picture 3.

Graph 1: DFS according to CEA expression (Kaplan Maier)

Cumulative Survival Curves

P = 0.05548
Gehan-Wilcoxon

Months

CEA –
CEA +
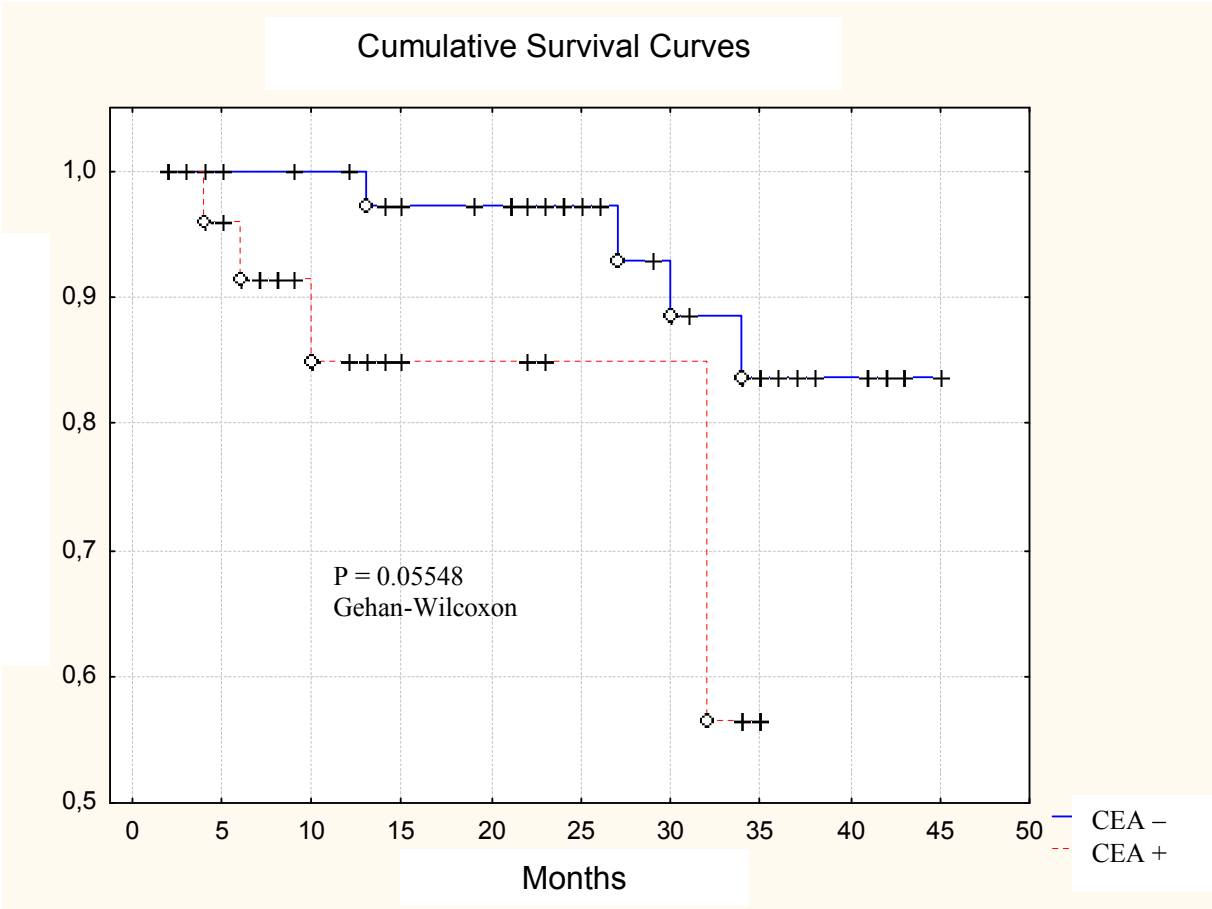
Picture 3: The example RT-PCR analysis record. Colored curves demonstrate fluorescence depending on amplification cycles. Early increase of fluorescence means more quantity of searched mRNA
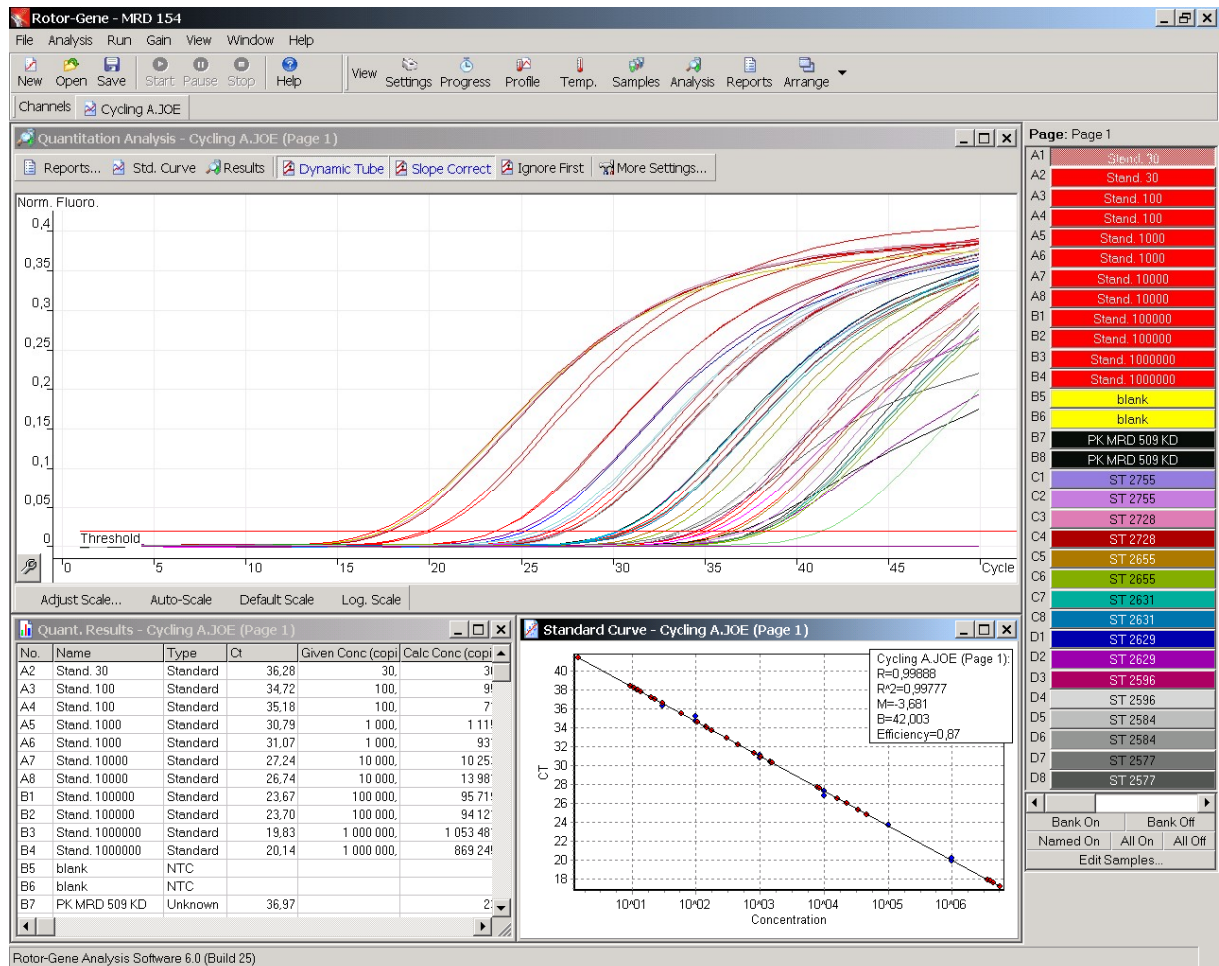

5.4. Immunocytochemical detection of minimal residual disease in bone marrow

Forty three patients were investigated. No occult cancer cells have been detected in first twenty eight patients. Therefore after that the investigational method has been modified using in-vitro cell cultures with subsequent immunocytochemical staining. Another 15 patients were investigated. Two of them had occult tumor cells in bone marrow (picture 4). Both samples were collected before the adjuvant treatment. In the first case there was no representative mRNA available for comparison. In the second case neither mammaglobin A nor B was identified. Likewise in all tested samples, CK 19 on RT-PCR has been identified. Because of concerns about reliability of the method that technique was eventually abandoned.

Picture 4:

Sample 1:

A/ Occult tumor cell detected using immunocytochemistry
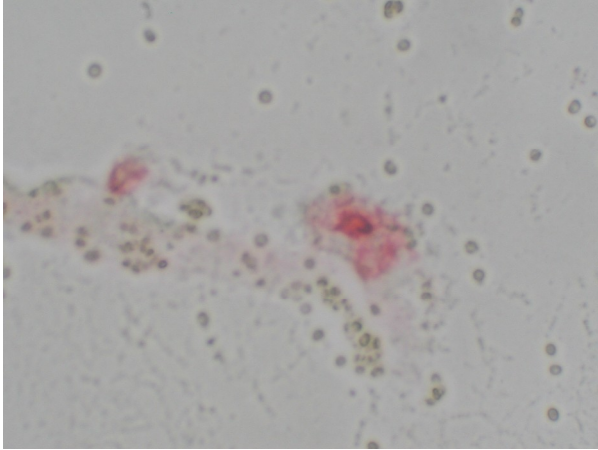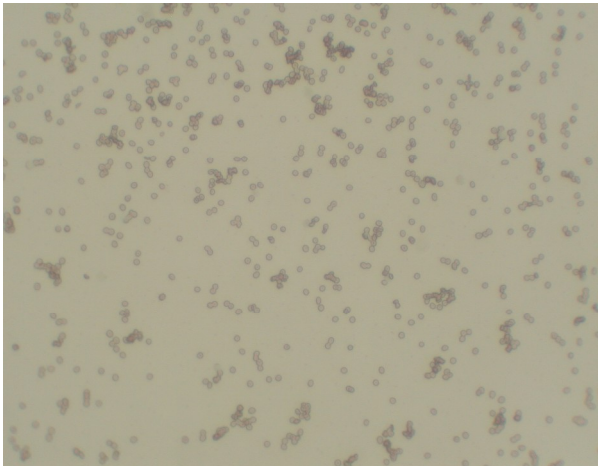
B/ Negative control

Sample 2:

A/ Occult tumor cell detected using immunocytochemistry

B/ Negative control
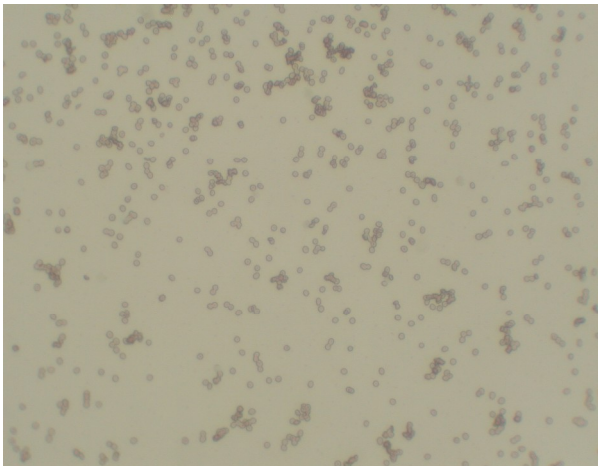
5.5. Immunohistochemical cytokeratin detection in axillary lymph nodes

Eleven patients out of 70 were investigated in this analysis. Thirty four were not included because they were node positive on routine histopathology. In remaining 25 out of 36 patients there was no adequate paraffin blocks available. Immunohistochemistry was performed using mouse antihuman monoclonal antibody A53-B/A2 against CK 19 (Immunotech ®). Minimal residual disease was detected in 1 out of 11 (9%) in 1 axillary

lymph node out of 9 investigated. There was no minimal residual disease in bone marrow. In this group there was no recurrence identified.

6. Discussion

Presence of occult tumor cells in bone marrow of patients with early breast cancer correlates with shorter DFS and OS (11-19,26-28,30,31,34-37,75-80). This evidence is not that firm for occult tumor cells in peripheral blood or lymph nodes (36,44,50,54,58,81,82,89-91,93,104,106,107,128-137). Cytokeratins as markers of minimal residual disease have been extensively studied. Disadvantage of cytokeratins is relatively low specificity (93,138) caused by possible illegitimate RNA transcription etc. (136,139,140). CK 19 has been mostly investigated. Some investigators explored CK 20 however this marker need not to be expressed in all breast cancer cells (138). CEA is a tumor specific marker nevertheless some breast cancers may lack its expression (141). Mammaglobin A is both sensitive and specific. On the contrary mammaglobin B is less specific and could be found also in salivary glands and uterus (142).

In our group of patients mammaglobin A was detected using nested RT PCR in 12% (6 out 51) patients before the treatment and in 8% (2 out 24) patients after the treatment. Three positively tested patients did not provide consent to have a second aspirate taken. In remaining 3 cases minimal residual disease was eradicated with adjuvant therapy. Mammaglobin A was also detected in 2 samples post adjuvant therapy in patients who were initially negative.

Mammaglobin B was identified in 4% (2 out 51) of pretreatment samples and in 8% (2 out 24) of post-treatment samples. The first patient with positive pretreatment sample did not provide consent for a follow up investigation. The second patient remained positive after the therapy. One patient was newly positively tested after treatment. Mammaglobin A and B were both detected in one patient in the pre-treatment sample (the follow-up sample was not collected) and in one patient in his post-treatment sample (newly detected mammaglobin A and persistence of mammaglobin B). There have been two similar studies investigating mammaglobin A expression published so far. Mammaglobin B has not been clinically tested yet. Ooka et al. (80) detected mammaglobin A transcripts in 33 (29.7%) out of 111 patients with breast cancers clinical stage I-III. After the median follow up of 21 months patients with bone marrow involvement had significantly shorter DFS (in fact DMFS) in both univariate

and multivariate analysis. Bossolasco et al (143) reported mammaglobin A in 9% of 22 patients which is a similar number like in our study. Prognostic significance has not been investigated.

The presence of CK 19 in all tested pretreatment samples was quite surprising. However some institutions published similar experience (93,139,141,144). Conflicting data in literature along with our own results suggested that RT PCR detection of CK 19 is unreliable.

In the subgroup investigated for mammaglobin A, B, and CK 19 only 2 recurrences have been reported to date (97% disease free survival). It makes all survival analysis extremely difficult to interpret. However mammaglobin A and with less confidence mammaglobin B seem to be useful markers of minimal residual disease. In the subgroup of pretreatment samples mammaglobin A expression positively correlated with higher grading, and higher number of infiltrated axillary lymph nodes. In the subgroup of post-treatment samples mammaglobin A expression negatively correlated with hormone receptor expression. Similar correlation was found between hormone receptor expression and mammaglobin B in both pretreatment and post-treatment group. Relatively lower prevalence of minimal residual disease in our population in comparison with other published studies (80,143,145) could be attributed to lower number of recurrences in our group of patients.

CEA expression has been investigated in lymph nodes, samples of peripheral blood, and bone marrow. Min et al. (146) used CEA RT-PCR detection in sentinel lymph nodes. Mitas et al. (107) reported similar results with quantitative RT-PCR. Other authors found CEA detection in the same indications less contributory (147). All studies enrolled small number of patients (from 17 to 22), which makes any clear cut conclusion difficult to draw. CEA detection in peripheral blood showed similar conflicting results (148). The mRNA CEA transcripts were detected in 17.4% to 67 % patients. Gerhard et al. (83) found CEA using RT-PCR in 4 out 6 patients with breast cancer. Zhong et al. (84) detected CEA in 27.8% of patients using the similar technique in the largest group of patients ever published (181 early breast cancer patients). Berois et al. (85) published the smallest proportion of positive findings of CEA in the bone marrow (17.4%; 8 out 46 patients). In our study we have identified minimal residual disease in bone marrow using quantitative CEA RT-PCR in 41% (29 out of 70) patients with early breast cancer.

In previously published studies immunocytochemically detected minimal residual disease was confirmed to be an independent adverse prognostic factor for shorter DFS and OS

in patients with early breast cancer (114). It has been supported by recently published meta-analysis (40).

CEA as a marker of tumor cells in peripheral blood was found as an adverse factor in patients with colorectal and non-small cell lung cancer (149,150). Jotsuka et al. (94) published a study with 101 early breast cancer patients and the presence of mRNA for CEA has been confirmed as an adverse prognostic factor. Stathopoulou et al. (151) investigated CEA expression in peripheral blood of patients with breast cancer, colorectal cancer and hematological malignancies. Unfortunately no survival data has been published. There has been no large study focusing on prognostic impact of CEA in bone marrow carried out. In our study after median follow up of 22 months 8 DFS events have been observed. Four had CEA transcripts in bone marrow. However 2 DFS events in patients with CEA transcripts were tumor unrelated. There was observed a trend towards shorter DFS in patients with CEA in the bone marrow (p=0.05548) which has not reached the level of statistical significance, however it has not been confirmed in DMFS and RFS analyses. DFS data should be interpreted with extreme caution, because results could be confounded by 2 tumor unrelated deaths in the group of patients with CEA expression in bone marrow. CEA expression has not correlated with other clinical and laboratory prognostic factors. The quantitative CEA RT-PCR detection is feasible and could be used in a future research of minimal residual disease in early breast cancer patients. Nevertheless the specificity of the method should be validated on bone marrow samples of healthy (or at least non cancer) volunteers in order to determine the level of illegitimate CEA transcription in normal bone marrow. The prognostic significance of CEA remains uncertain.

Because of unsatisfactory results with immunocytochemistry using published technique (108) the method of bone marrow cells in-vitro cultures was explored. Occult tumor cells were identified in 2 patients out of 15 (13%) in pretreatment samples. Investigated samples were exposed for a significant period of time to in-vitro condition therefore reliability of the method is questionable.

Currently the most promising area for the use of minimal residual disease is an investigation of lymph nodes or sentinel lymph nodes. Minimal residual disease could lead to more accurate diagnosis in terms of possible infiltration. Nevertheless, at the moment occult tumor cells diagnosed on immunocytochemistry in a sentinel lymph node do not warrant upstaging in terms of TNM classification (45), because prognostic significance remains

uncertain. In our study patients with known axillary infiltration have not been investigated. Minimal residual disease on immunohistochemistry was detected in 1 patient in 11 investigated (9%). In this subgroup no recurrence was observed therefore prognostic impact has not been addressed.

Our results may contribute to better understanding of the role of new markers such as mammaglobin A, B and CEA in diagnosis of minimal residual disease in breast cancer. Likewise in some previously published reports CK 19 failed to prove it's diagnostic utility (93,139,141,144).

7. Conclusion

Breast cancer is one of the most serious health problems in our society. In the Czech Republic there are nearly 6000 women newly diagnosed annually. Despite the increasing incidence the mortality is leveling off or even decreasing in many countries (152,153). It is probably attributed to earlier diagnosis and the introduction of screening mammograms in many developed countries (154), and new findings in molecular biology of tumors. Several molecular factors are already routinely used in routine clinical practice as prognostic (estrogen and progesterone receptors, HER-2/neu, p53, Ki-67, vascular endothelial growth factor-VEGF), and predictive factors (estrogen and progesterone receptors, HER-2/neu) or therapeutic targets of anticancer treatment (estrogen and progesterone receptors, EGFR, HER-2/neu, HER3, HER4, VEGF, mTOR) (7,45,155-157).

The detection of minimal residual disease in early breast cancer is another attempt to implement modern diagnostic technologies in order to improve treatment outcomes.

The aim of the study was to investigate diagnosis and prognostic implications of minimal residual disease in axillary lymph nodes, and bone marrow of patients with early breast cancer.

The most promising material was bone marrow. From the clinical point of view it is necessary to validate both immunohistochemistry and RT-PCR examination of axillary lymph nodes in order to achieve more accurate interpretation of sentinel lymphadenectomy.

In our study minimal residual disease in bone marrow was detected in 4-41% patients before a neoadjuvant or an adjuvant therapy depending on marker (mammaglobin A, mammaglobin B, CEA) used and the pantients' subgroup. After an adjuvant treatment minimal residual disease (using mammaglobin A, mammaglobin B) was detected in 8% of

patients. The prognostic significance of minimal residual disease detected with RT-PCR remains uncertain.

Minimal residual disease in lymph nodes was confirmed on immunohistochemistry in 9% of patients. These results are difficult to interpret due to small number of patients investigated.

A classical chemotherapy has probably very little potential for a further significant improvement of treatment outcomes. Therefore it is absolutely necessary to better understand key molecular mechanisms leading to the onset of malignant disease. Currently there is bustling development of new diagnostic tests and new drugs which significant differs from classical chemotherapy. They are targeted against precisely defined cell targets involved proliferation, DNA repair or apoptosis. Some of these drugs are already used in clinical practice, others in final stags of clinical staging. I believe this study brought closer experimental approaches to a routine clinical practice.

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