The Adjuvant Therapy Influence upon Micrometastasis in Bone Marrow Aspiration and Detection of Relapse Rate in Breast Cancer Patients Follow up

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Abstract

Introduction: Breast cancers in the early phase frequently undergo distant metastasis and survival of patients is greatly dependent on distant metastasis. The occurrence of micrometastasis has been suggested to relate with prognostic features of breast cancer, such as lymph node metastasis and the presence of vascular invasion. The aim of this study was to examine the presence of keratin-19 mRNA of epithelial tumors in bone marrow aspirates obtained from breast cancer patients and its possible correlation with tumor staging and disease free survival.

Methods and materials: Bone marrow samples were obtained from 53 breast cancer patients at the time of adjuvant therapy after surgery. We separated the mononuclear fraction from the samples and carried out nested reverse transcriptase polymerase chain reaction (RT-PCR) for the detection of keratin-19 mRNA with two different pairs of primers. After adjuvant therapy, patients undergoing Bone marrow aspiration and repeat separation the mononuclear fraction from the samples and carried out nested reverse transcriptase polymerase chain reaction (RT-PCR) for the detection of keratin-19 mRNA with two different pairs of primers, the patients were followed up at 3-month intervals.(1,2) We studied the possible correlation of the detection of keratin-19 mRNA with tumor size, nodal involvement, stage and recurrence rate, overall survival and disease- free survival.

Results: We studies 53 Breast cancer patients (Stage 1-11I) for presence of Bone marrow micrometastasis by nested-PCR for cytokeratin 19. Before adjuvant chemotherapy it was positive in 17 patients (32%). Presence of DTC was independent to clinical stage.

We followed 15 out of 17 bone marrow positive, by the same methods for presence of micrometastasis after adjuvant chemotherapy and observed that 4 patients remained positive after adjuvant chemotherapy.

Conclusion: With a median follow up of 272 days three metastasis developed, in our cohort which all happened in bone marrow positive patients. Our study is ongoing to increase the number of patients, longer follow up and also to compare peripheral blood with bone marrow.

Keywords: Micrometastasis, Bone Marrow Aspiration, Breast Cancer, Relapse Rate

Introduction

Early distant metastasis is fairly common in breast cancers and recurrence and survival are greatly influenced by the distant metastasis. To minimize recurrence in distant sites, chemotherapy and hormone therapy Target therapy for example Herceptin for Her2neu positive patients are commonly used. Major prognostic factors are nodal status, tumor size, histological grade and hormonal receptor status. Theoretically, distant metastasis or recurrence is the result of tumor growth from micro metastasis already

existing at the time of surgery. Therefore, many efforts have been made to uncover micro metastasis in distant organs to characterize the cancer biologically and to determine sub clinical staging. Thus far, two methods have been used for' this purpose: one of them is the immunochemical staining of the nucleated cells from bone marrow with epithelial markers and the other is the detection of epithelial mRNA markers from the marrow cells with the reverse transcriptase polymerase chain reaction (RT-PCR) technique.(4,5,6) We report here the results of our prospective study on clinical significance of bone marrow micro metastasis detected with nested RT-PCR for keratin-19 mRNA in breast cancer patients.

Patients and materials

During the period between 2003 and 2005, 53 female patients were diagnosed as having breast cancer and were treated at Day Hospital, Shariati Hospital, Tehran, IRAN, consented to our prospective study and were enrolled .These patients had undergone breast operation and bone marrow aspiration from the iliac spine before and after adjuvant chemotherapy, and subjected to follow-up studies similarly to other breast cancer patients, regardless of the results of the bone marrow RT-PCR study. The median follow-up time was 272 days. The clinical and pathological data and the results of the bone marrow keratin-19 nested RT-PCR were reviewed and analyzed together with clinical results on recurrence and survival. Total RNA was extracted from bone marrow, using guanidine thiocyanate–phenolchloroform. Keratin-19 mRNA was amplified by RT-PCR using two pairs of primers. The final product was separated in a 2% agarose gel containing ethidium bromide.

RNA Isolation: A 8 ml amount of bone marrow obtained from breast cancer patients was carefully layered over 10 ml of Lymphoprep (Nycomed). Nucleated cells were spun down at 2700 r.p.m. for 20 min and stored at –70°C until used. These cells were dissolved in TRIzol reagent (Gibco BRL) and RNA was isolated according to the manufacturer's instructions and quantified spectrophotometrically at 260 nm.

RT-PCR and Nested PCR: A 30 µI RT-PCR reaction volume contained 50 mM KC1, 10 mM Tris-HCL (PH 9.0), 0.1% Triton X-100, 200 µM each of dNTP, 2.5 mM $MgC1_2$, 0.1 mM DTT, 60 U M-MLV reverse transcriptase, 0.5 U of tag polymerase, 12 U of RNase inhibitor, 10 mM external sense primer, 10 mM external antisense primer, I ug of total RNA and DEPC-DW. Complementary DNA was made at 42°C for 60 min. The cycle conditions were as follows: 2 min at 95°C, 35 cycles of 1 min at 94°C (denaturation), 1.5 min at 57°C (annealing), 2 min at 72°C (extension) and lastly 10 min at 72°C. A 2µl volume of the first amplification product was mixed with 18 µl of the second PCS buffer containing 50 mM Kcl, 10 mM Tris–HC1 PH 9.0), 0.1% Triton X-100, 200 µM each of dNTP, 2.5 mM $MgC1_2$, 0.5 U of tag polymerase, 10 mM internal sense primer, 10 mM internal antisense primer and DEPC-DW. The reaction cycle was the same as described above. Thermal cycling was performed in a GeneAmp 9600 apparatus (Perkin-Elmer). Each sample was subjected to electrophoresis with 2% agarose gels, stained with ethidium bromide and visualized on a transilluminator. The presence of intact RNA was confirmed by RT-PCR using β-actin-specific primers. To determine the sensitivity of RT-PCR assays, 10^6peripheral blood mononuclear cells obtained from a normal donor were mixed with decreasing numbers of MCF-7 cell lines.

Patient Surveillance and Statistics: we evaluated recurrence of the disease by physical examination, serological testing, chest PA, abdominal ultrasonography and bone scans at 3 month intervals. Disease-free survival comparisons between groups with CK-19 positive and negative results were conducted using the Kaplan–Meier test. Disease-free survival comparisons between each stage were performed in the same manner. Values of $P < 0.05$ were considered statistically significant. Multivariate analysis was not conducted owing to the small patient numbers.

The information of our patients showed in tables- 1, 2, 3, 4.

Results

The sensitivity of the assay appeared RT-PCR to be highly satisfactory , There for so that nested RT-PCR could detect 1 breast cancer cells in one million nucleated blood cells.

Table- 1. T tumor of patients.

T	Percent	N	Percent
T1b	3.8	N0	51.9
T1c	30.8	N1	28.8
T2	57.7	N2	15.4
T3	7.7%	N3	3.8
Total	100	Total	100

Table- 2. Age, Follow up time

*The midum follow -up time was 271 days (Min:127,Max :412)
*Mean age: 46yr 31.4% >51 yr
*Female: 52cases
*Rt breast:53.5%

Table 3:Grade, Lymph invasion, perineu, vascular

Grade I: 2.4%
Vascular 81.6%
Grade II: 41.5%
Grade III: 56.1%
Lymph invasion: 75%
Vascular: 81.6%
Perineu: 50%

Table- 4. Hormon receptor, cathepsine, Her 2neu, ki 67, P 53, Tumor Necrosis

Hormon receptor: Er & Pr:62.3%
Cathepsine : 20.8
Her 2neu: (3^+) 16%, (2^+) 26%
Ki67: 53.2%
P53: 43.1%
Tumor Necrosis: 52.3%

Er: Estrogen receptor, Pr: Progestrone receptor

Table- 5: Results of CK-19 in our patients

BMA positive for CK-19	Pretreatment BM Negative for CK-19
32%	68%
17pts	36pts
BMA positive for CK-19 After treatment: in four of fifteen patients	

Table- 6. Relationship between the results of RT-PCR and

Relationship between RT-PCR and T;	P values
T tumor & BMA+ for CK-19	0.6
Stage & BMA for+ CK-19	0.6
LND & BMA for+ CK-19	>0.05

Results

The sensitivity of the assay appeared RT-PCR to be highly satisfactory, there for so that nested RT-PCR could detect 1 breast cancer cells in one million nucleated blood cells. There were 17.6 percent cases positive among patients with Stage 52.9 percent cases positive among patients with stage II, 29.4 percent cases with stage III.(Table- 5)

According to node staging, 29.4 percent cases were positive among patients with N2 and N3, 41.2 percent cases in patients with NO and 27.4 percent cases in patients with N1. However, no statistical significance was found between bone marrow micro metastasis and tumor size, lymph node metastasis and stage.(9, 10, 11) As shown in Table- 6. No statistical significance was found between bone marrow micro metastasis and Stage; grade .Vascular; hormone receptore; Her 2neu; P 53; Ki 67; Cathepsine; Locally advanced.

Results of RT-PCR and Recurrence: Table- 6 shows that there were three cases of recurrence among 53 patients, one case of recurrence in patients with StageI, two cases of recurrence in patients with stage II. According to lymph node metastasis, there were one cases of recurrence in patients with N0, one in patients with NI and one in patients with N2.(4) There was no recurrence in patients stage I, one cases of recurrence in patients with stage III and two cases of recurrence in patients with stage II. All three cases of recurrence had RT-PCR positive for keratin-19 mRNA and there was no recurrence in patients with RT-PCR negative for keratin-l9 mRNA.

After follow-up, we found a significant relationship in survival curves between bone marrow micro metastasis and recurrence (P=0.03). However, tumor size, lymph node involvement and hormonal receptor did not affect survival curves, as shown in disease-free survival curve in our patients.

Discussion

The most important prognostic factor of breast cancer is lymph node metastasis.

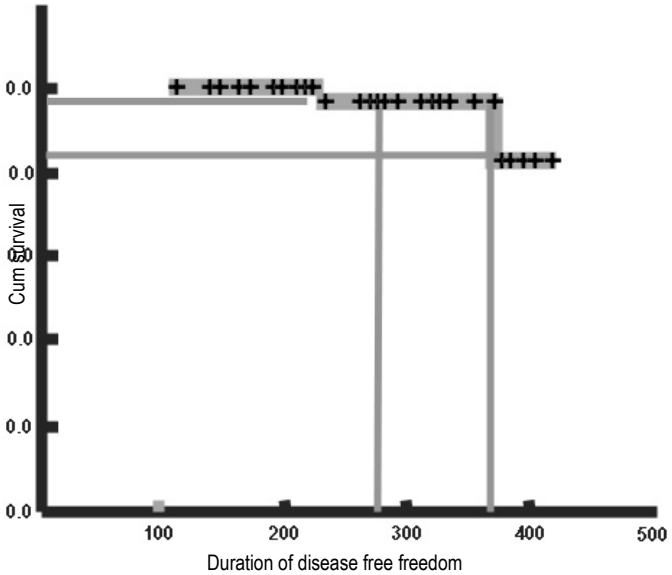

Figure- 1. disease-free survival curve

Among the biological characteristics of breast cancer, lymph node metastasis is closely related with distant metastasis and long-term survival. Thirty percent of breast cancer patients (one patient) without lymph node metastasis have been shown to have tumor recurrence within follow up. Among these patients, therefore, it is extremely important to screen out those who should receive adjuvant chemotherapy. Recently, there has been a strong tendency to apply adjuvant chemotherapy with smaller breast cancers, and the majority of the breast cancer patients take the adjuvant chemotherapy. In breast cancer patients, there is frequently an early distant metastasis without local recurrence, but with many bone marrow metastases. There have been many studies to unravel micrometastasis in bone marrow, because of the strong tendency for bone marrow invasion in these patients. As a marker of bone marrow metastasis in this study, we used keratin-1 9, which is only expressed in epithelial cells and not in normal bone marrow cells. Keratin-I9 has been widely studied as a marker of micrometastasis, because it has a high specificity for breast cancer cells and reasonable specificity for bone marrow and blood. RT-PCR is a method to determine proteins to find a specific protein that is contained in a specific cell. mRNA is first converted into cDNA and then multiplied. Schoenfeld et al. first employed RT-PCR to detect axillary lymph node metastasis of cancer(3, 4, 5) and Kruger et al. reported bone marrow metastasis in 14 cases among 24 breast cancer patients.(2) Vannucchi et al. reported that breast cancer cells were found among stem cells which were used in peripheral blood stem cell transplantation (PBSCT) after high-dose chemotherapy(3) and some patients treated with PBSCT showed early cancer recurrence, suggesting that patients with micrometastasis to bone marrow have greater chance of cancer recurrence.(3) Recently, however, Braun et al. reported that micrometastasis to bone marrow is an independent prognostic factor after immunohistochemical study of 150 patients.(4)

In the last decade, faster and more precise quantitative and real time RT-PCR methods have been developed.(5,6) However, the existence of keratin-19 pseudogene leads to false positives: this pseudogene shows an amino acid sequence similar to that of keratin-19 and can be expressed in non-epithelial tissue.(7,8)

Further, the nested RT-PCR used in this study had high sensitivity owing to the use of repeated PCR. Recent studies on breast cancer micrometastasis using the nested RT-PCR method indicated 42-49% micrometastasis to bone marrow, showing a higher positive rate than in studies carried out by,RT-PCR.(9,10) Our results showed a 32% positive rate, i.e. a higher sensitivity than the other methods. However, it is very difficult to explain this difference because our results for the sensitivity test were similar to the others (only one cancer cell could be found among, 10^6 cells).

Nevertheless, this result might have some significance, since early recurrence of breast cancer has been observed only in the group positive to keratin-19.

In this study, patient treatment and follow-up were carried out in the same manner without any consideration of the RT-PCR results. Comparing various important prognostic factors for micrometastasis to bone marrow, cancer recurrence in stages 2 and 3 was found to be a reasonable prognostic factor. It is well known that lymph node metastasis and expression of hormone receptor and pathological characteristics are important prognostic factors.(11) However, in our study there was no statistically significant difference in patient survival. This might have been due to a low recurrence rate and fewer cases. Further studies should be carried out to develop effective guidelines for treatment by reducing the false-positive rate.

In conclusion, the nested R.T-PCR of keratin-19 for micrometastasis of breast cancer showed high sensitivity for micrometastasis of bone marrow. Every early recurrent breast cancer patients in the present study showed a positive reaction in nested RT-PCR with statistical significance. To be useful as an independent prognostic factor of nested RTPCR in breast cancer patients, further studies with more cases and longer-term follow-up are required.

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