Abstract

The detection of circulating tumor cells may have important prognostic and therapeutic implications. It is our hypothesis that circulating breast cells can be detected, isolated and characterized from breast cancer patients. Fifteen highly progressive metastatic breast cancer patients were analyzed for this study. Using conventional methods to detect for circulating tumor cells does not enable us to account for the cancer cells at low concentrations. However, new molecular techniques have been developed to detect for occult tumor cells. In this study, the breast tumor cells were isolated from the blood using a novel sensitive immunomagnetic method from the mononuclear fraction isolated from leukapheresis. Nested RT-PCR for mammaglobin mRNA was performed. All fifteen patients contained detectable levels of actin mRNA; CK-19 mRNA was observed in ten patients and mammaglobin mRNA was observed in four patients with the CK-19 mRNA. We have been able to isolate circulating breast cells from the bloodstream using a novel immunobead technique for microscopic evaluation by RT-PCR. The use of multiple markers greatly enhances the molecular detection of circulating breast cells. Mammaglobin and Cytokeratin-19 seem to play a critical role in the proliferation of breast cancer. This technique can be used for molecular characterization of the circulating tumor cells in breast cancer and other solid tumors. This would help assess, stratify, and design targeted therapy for cancer patients.

Introduction

Cancer is often referred to as “the silent killer.” At the onset of cancer, cancer cells are difficult to identify and by the time they have been detected, not much can be

done to cure the patient[1]. Cancer cells are characterized as abnormal cells that divide and multiply at an uncontrollable rate. Detecting circulating tumor cells at low concentrations could aid in the diagnosis and treatment of cancer patients[2].

Tumors may develop in any organ of the body. Tumor cells multiply, metastasize and evade other organs of the body. These tumor cells travel in the body through the lymphatic system or bloodstream. As the cancer progresses, the tumor becomes more visible because of its growth[3]. Breast tumors are clinically confined to the breast and neighboring lymph nodes[4].

Various treatments are available for patients to arrest the progression of the tumor and mitigate its damaging effects. Individuals with cancer may have primary surgery, where the tumor is physically removed. They may also undergo adjuvant or neoadjuvant chemotherapy where anticancer drugs are used to suppress cancer progression[5]. Biological therapy can be used to stimulate cells to restore their ability to fight infections and other diseases. Radiation therapy is an alternative option; it uses high-energy radiation from computerized axial tomography (CAT scan), magnetic resonance imaging (MRI), gamma rays, neutrons and other sources to kill cancer cells and shrink tumors[6].

Tumor markers are branches in specific cells that can often be detected in higher-than-normal amounts in the blood of patients with a certain type of cancer. There is a specific molecular marker of every type of cell. These markers differentiate, characterize,

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[1] Johnson, P. W. M., Burchill, S. A., and Selby, P. J. The molecular detection of circulating tumor cells. Br. J. Cancer, *72:* 268–276, 1995.
[2] Ghossein RA, Bhattacharya S. Molecular detection and characterization of circulating tumour cells and micrometastases in solid tumours. Eur J Cancer. 2000 Aug 36 (13 Spec No):1681-94.
[3] Fellowes VS, Husebekk A, Gress RE, Vance BA. Minimal residual disease detection in breast cancer: improved sensitivity using cytokeratin 19 and epidermal growth factor receptor RT-PCR. Int J Oncol. 2004 Apr; 24(4):861-7.
[4] Ghossein RA.
[5] Ghalie, R. The Osborne/Rosen article reviewed. Oncology, *8:* 36–40, 1994.
[6] Ghossein RA.

and distinguish one cell from another. They are located on the surface of cells and are composed of protein molecules. Molecular markers for breast cells are MUC 1, CEA, mammaglobin and cytokeratin-19[7,8]. Tumor markers are produced by the tumor itself or by the body in response to the presence of cancer or non-cancerous conditions. Tumor marker levels can be useful in the detection and diagnosis of different types of cancer[9].

Methods and Procedures

Highly progressive metastatic breast cancer patients were analyzed for the study. All the patients were positive for metastases by imaging, bone scan and CT scan. A group of healthy individuals with no apparent malignancies were taken as negative controls. Informed consent from all the patients prior to participation in the study was obtained. The peripheral blood was mixed with appropriate amount of phosphate buffered saline (PBS) and separated using Ficoll gradient by density gradient centrifugation. The mononuclear and a portion of the granulocytic layer were taken for further study.

Extraction of Circulating Tumor Cells Using Immunomagnetic Beads

One half of the mononuclear cell fraction isolated from the blood samples was incubated with magnetic beads coated with Ber-EP4 antibody directed against human epithelial antigen, a membrane antigen widely expressed in epithelial cells. The other half of the mononuclear cell fraction (used for microscopic evaluation) was incubated with magnetic beads coated with Ber-EP4 antibody linked to the magnetic beads via a DNA linker. The magnetic cell fraction was isolated and treated with DNase to remove the

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[7] Ronald A. Ghossien, Leo Carusone, and Satyajit Bhattacharya: Molecular detection of micrometastases and circulating tumor cells in melanoma prostatic and breast carcinomas. In vivo 14: 237 – 250, 2000.

[8]Jean-Charles Soria[1], Laurent R. Gauthier[1], Eric Raymond, Christine Granotier, Luc Morat, Jean-Pierre Armand, François D. Boussin[2] and Laure Sabatier Molecular Detection of Telomerase-positive Circulating Epithelial Cells in Metastatic Breast Cancer Patients. Clin. Cancer Res., August 1, 2003; 9(8): 3004 - 3011.

[9] Singletary, S. E., Larry, L., Tucker, S. L., and Spitzer, G. Detection of micrometastatic tumor cells in bone marrow of breast carcinoma patients. J. Surg. Oncol., *47:* 32–36, 1991.

magnetic beads from the cells. The isolated immuno-magnetic bead-free cells were then cytospun, fixed in Methanol: Acetone (1:1), and stored at -80°C. Messenger RNA was isolated using Oligo (dT) attached to magnetic beads after epithelial cell enrichment. The isolated RNA was stored at -80°C until further used[10,11].

Reverse Transcriptase-Polymerase Chain Reactions

Before the addition of RT-PCR reagents, twenty microliters of the Oligo-dT-mRNA-complex solution was heat denatured in a 65°C water bath for 5 minutes and immediately chilled on ice. The 50 uL total RT-PCR volume consisted of 1X primer, 0.2 mM/l each deoxyribonucleotide triphosphaphate (Perkin Elmer). The RT reaction and the first round of PCR amplification were performed sequentially in a single tube without interruption in a Perkin Elmer 9600 PCR thermocycler, using the condition for each primer. For the second round of PCR, 2 uL of the first round PCR product was added to 18 uL of DEPC-treated water, heated in 65°C and then immediately chilled on ice before the addition of PCR reagents. Except for the presence of RT, the reagents concentrations in the 50 uL total PCR volume were similar to the concentrations used in the first round of the PCR amplification[12].

RNA isolated from MCF-7 (breast carcinoma cell line) and carcinoma tissues from breast cancer patients were used as positive controls. Negative control PCR

[10] Pelkey TJ, Frierson HF and Bruns DE: Molecular and immunological detection of circulating tumor cells and micrometastases from solid tumors. Clin Chem 42:1369 – 1381, 1996

[11] Ronald A. Ghossien, Leo Carusone, and Satyajit Bhattacharya: Molecular detection of micrometastases and circulating tumor cells in melanoma prostatic and breast carcinomas. In vivo 14: 237 – 250, 2000

[12] Datta, Y. H., Adams, P. T., Drobyski, W. R., Ethier, S. P., Terry, V. H., and Roth, M. S. Sensitive detection of occult breast cancer by the reverse-transcriptase polymerase chain reaction. J. Clin. Oncol., *12:* 475–482, 1994.

consisted of reaction mixtures containing all the reagents but without any template. MCF-7 was used to define the sensitivity of the assays[13].

CK-19 RT-PCR

For nested RT-PCR analysis of the cytokeratin-19 (CK-19) mRNA, 20 uL of the Oligo-dT-mRNA-complex was subjected to a 50 uL RT-PCR reaction containing reagents. The primer sequence was obtained from Dynal Inc. Twenty pM of outer forward (5'-GTGGAGGTGGATTCCGCTCC-3'), outer reverse (5'-TGGCAATCTCCTGCTCCAGC-3'), inner forward (5'-ATGGCCGAGCAGAACCGGAA-3') and inner reverse (5'-CCATGAGCCGCTGGTACTCC-3') were used in the same reaction mixture. The primers were shown to span the intronic sequences and have significant mismatch with any of the pseudo genes (Dynal). Reactions were first incubated at 42°C for 15 minutes and then at 97°C for 15 seconds (RT reaction). Afterwards, we heated the primers at 94°C for 5 minutes, subjected to 30 cycles of 74°C for 30 seconds, 58°C for 30 seconds and finally 72°C for 7 minutes[14].

Actin RT-PCT

To confirm for the presence of RNA, 20 ml of the Oligo-dT-mRNA sample from each sample were subjected to single tube RT-PCR for actin mRNA using previously published primers. The PCR conditions and reagents concentrations were identical to those of Ben-Erza et al except for the RT reaction, which was carried out at 42°C for 15

[13] Noguchi, S., Aihara, T., Nakamori, S., Motomura, K., Inaji, H., Imaoka, S., and Koyama, H. The detection of breast carcinoma micrometastases in axillary lymph nodes by means of reverse transcriptase polymerase chain reaction. Cancer (Phila.), *74:* 1595–1600, 1994

[14] Foss AJ, Guille MJ, Occleston NL, Hykin PG, Hungerford JL, and Lightman S. The detection of melanoma cells in peripheral blood by reverse transcriptase polymerase chain reaction. Br J Cancer 72: 155 – 159, 1995.

min. using 5 units of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim) and followed by a 97°C step for 15 seconds. The 154-bp PCR product generated from actin mRNA was detected by agarose gel electrophoresis[15].

Mammaglobin RT-PCR

For nested RT-PCR analysis of mammaglobin mRNA, 20 uL of the Oligo-dT-mRNA-complex was subjected to a 50 uL RT-PCR reaction containing reagents. Twenty-five pM of outer forward (5'-AGCACTGCTACGCAGGCTCT-3'), outer reverse (5'ATAAGAAAGAGAAGGTGTGG-3'), inner forward (5'-GAGGTGGATTCCGCTCCGGGCA-3') and inner reverse (5' ATCTTCCTGTTCCCTCGAGCAG-3') were used in the same reaction mixture. The following thermo-cycling conditions were used: 42°C for 15 minutes and then 97°C for 15 seconds (1 cycle) in method 1 (RT reaction); followed by 40 cycles of 45 seconds at 94°C for template denaturation; 60 seconds. at 55°C for primer annealing; and 45 seconds at 72°C for primer extension; and a final primer extension of 17°C for 10 minutes. The second round of PCR used a 1:50 dilution of the primary PCR reaction and 25 pmol of the inner forward and inner reverse primers. The thermo-cycling conditions for the second PCR reaction were: 94°C for 45 seconds, 58°C for 60 seconds, 73°C for 45 seconds, for 45 cycles, with a final primer extension at 72°C for 15 minutes. The nested RT-PCR reaction yielded a product of 330 bp[16].

[15] Foss AJ, Guille MJ, Occleston NL, Hykin PG, Hungerford JL, and Lightman S. The detection of melanoma cells in peripheral blood by reverse transcriptase polymerase chain reaction. Br J Cancer 72: 155 – 159, 1995.
[16] Foss AJ

Gel Electrophoresis

The PCR amplified products were run on a 2% ethidium bromide agarose gels and observed under a UV transilluminator. To confirm the presence of amplifiable RNA, every sample was subjected to RT-PCR for actin mRNA and confirmed on agarose gel electrophoresis[17].

Data and Observations

Sample #	Actin	Cytokeratin 19	Mammaglobin
1	+	+	-
2	+	+	+
3	+	-	-
4	+	-	-
5	+	-	-
6	+	+	+
7	+	+	+
8	+	+	-
9	+	+	-
10	+	-	-
11	+	+	+
12	+	-	-
13	+	+	-
14	+	+	-
15	+	+	-
Percentage	**100%**	**67%**	**27%**

Positive Result: +
Negative Result: -

[17] Ghossein RA, Bhattacharya S, Coit DG. Reverse transcriptase polymerase chain reaction (RT-PCR) detection of melanoma-related transcripts in the peripheral blood and bone marrow of patients with malignant melanoma. What have we learned? Recent Results Cancer Res. 2001; 158:63-77

After running the gel electrophoresis, we observed that 15/15 patients tested positive for Actin, 10 patients had the presence of cytokeratin-19 and 4 patients had the presence of mammaglobin.

Results and Discussion: RT-PCR

Fifteen breast carcinoma patients at terminal stages with lymph node metastases were used for the study. Ten of the patients were found to be positive for CK-19 mRNA (67%). Four of the patients were also positive for mammaglobin mRNA (27%) and CK-19 mRNA as detected by RT-PCR; this makes up all of the 40% co-expressed cases.

In this experiment, we had looked for the presence of Actin, CK-19 and mammaglobin, respectively. The presence of Actin indicates whether we have collected sufficient amounts of RNA to carry out the experiment. CK-19 is a protein marker associated with breast or other forms of cancer, and its presence signals the onset of a disease[18]. This marker is not specific enough to determine the exact type of cancer a patient has. But it shows that the patient does indeed have cancer. After testing for CK-19 a more specific marker was used to determine the type of cancer the patient has. The third protein marker, mammaglobin is limited to the mammary epithelium and would be present only if a person develops breast cancer.

Mammaglobin RT-PCR was not imposed on the five samples which were negative for the cytokeratin-19 marker. The reason for this is because mammaglobin is a much more specific marker compared to CK-19. Therefore, when samples 3, 4, 5, 10 and 12 were tested negative, we assumed that mammaglobin would be the same. Thus the presence of mammaglobin was not tested in these samples.

[18] Fellowes VS, Husebekk A, Gress RE, Vance BA. Article Title: Minimal residual disease detection in breast cancer: improved sensitivity using cytokeratin 19 and epidermal growth factor receptor RT-PCR. Int J Oncol. 2004 Apr; 24(4):861-7.

Several specific markers have been used as surrogate markers to detect occult epithelial malignancies. A nested RT-PCR assay for carcinoembryonic antigen (CEA) was to identify metastases in histologically negative lymph nodes from patients with breast cancer. Many similar assays based on cytokeratin 20 and keratin 19 have been used to detect occult breast tumor cells in lymph nodes and the peripheral circulation. These molecular assays demonstrate enhanced sensitivity over serial sectioning and immuno-histochemical approaches[19]. However, they are plagued by low specificity and their utility is often limited by a low level of keratin gene expression normally present in lymphoid and other non-epithelial cells[20]. We have demonstrated in this work that the use of primers in cytokeratin-19 detection is not only highly sensitive but also extremely specific. No false positive results were detected using these conditions in assays described here[21].

High levels of mammaglobin mRNA had been observed previously, in primary metastatic and occult breast tumors[22]. In this study, mammaglobin expression was evident in all the cases which were positive for the presence of cytokeratin (40% in co-expressed cases), there implying that in some of the patients mammaglobin in transcribed with cytokeratin, whereas, in some only cytokeratin is expressed (60% in cytokeratin-only cases).

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[19] Fellowes VS, Husebekk A, Gress RE, Vance BA. Article Title: Minimal residual disease detection in breast cancer: improved sensitivity using cytokeratin 19 and epidermal growth factor receptor RT-PCR. Int J Oncol. 2004 Apr; 24(4):861-7.

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[21] Bostick, P. J., Chatterjee, S., Chi, D. D., Huynh, K. T., Giuliano, A. E., Cote, R., and Hoon, D. S. Limitations of specific reverse transcriptase polymerase chain reaction markers in the detection of metastases in lymph nodes and blood of breast cancer patients. J. Clin. Oncol., *16:* 2632–2640, 1998.

[22] Courtemanche, D. J., Worth, A. J., Coupland, R. W., and MacFarlane, J. K. Detection of micrometastases from primary breast cancer. Can. J. Surg., *34:* 15–19, 1991.

Conclusion

It is possible to isolate circulating breast carcinoma cells using a novel immunobead technique for RT-PCR. Multiple marker RT-PCR assays are superior to single marker in the detecting CTC in breast carcinoma. This experiment demonstrates that mammaglobin expression is a promising marker for neoplastic breast epithelial cells. It also provides sufficient evidence to warrant larger clinical studies using mammaglobin along with cytokeratin-19 as a molecular marker for early detection. Through early detection, staging prognosis and/or relapse monitoring of breast cancer could be more effective and efficient[23].

[23] Redding, W. H., Coombes, R. C., Monaghan, P., Clink, H. M., Imrie, S. F., Dearnaley, D. P., Ormerad, M. G., Sloane, J. P., Gazet, J. C., Powles, T. J., and Munro Neville, A. Detection of micrometastases in patients with primary breast cancer. Lancet, *2:* 1271–1273, 1983.

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Ghalie, R. The Osborne/Rosen article reviewed. Oncology, *8:* 36–40, 1994.

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