Multiple mRNA markers for detecting circulating tumor cells in breast cancer patients

Abstract

The detection of circulating tumor cells (CTCs) is suggested to be a potential method in early detection of cancer. Aimof this study is to employ the membrane array to develop a panel of mRNA markers for detectingCTCs in breast cancer patients.Twenty genes with highly differentiated expression were selected from microarray studies. The subsequent validation analysis in 30 pairs of breast tissue samples showed that 19 genes had more than 2-fold over-expression in cancer tissues compared with those in normal tissues in 80% paired samples. Furthermore, membrane-array experimenton blood samples of 64 normal controls and 87 breast cancer patients were conducted, and the predictive power of each gene was evaluated by analyses of the gene expression level using receiver operating curve (ROC). Among the 20 genes, 8 genes that had the largest area under the curve (AUC) beyond 0.8were selected as the diagnostic markers. According to the ROC analysis, when setting the cut-off point as 5 positive genes, the gene array of 8 markers couldbe recognizedas positive with the sensitivity and specificity of 90% and 89%respectively. These results suggest that themRNA marker arraycould be useful for detecting CTCs of breast cancer patients.

Keywords: mRNAmarker, circulating tumor cells, Breast cancer

Introduction

The incidence and the death rate of breast cancer are at the first and fourth stage respectively in Taiwanese women[1], which seriously threaten the health of women. Early detection is one of the most effective means of reducing cancer mortality. Through appropriate treatment, the 5-yearsaverage survival rate of breast cancer can reach to 88% in stage 1 and 93% in stage 0 [2]. Therefore, the breakthrough diagnostic method of breast cancer is expected to decrease the death rate of breast cancer.

The most convenient, side-effect free and acceptable detecting method for clinical breast cancer diagnosis and postoperative follow-up is using peripheral blood samples. However, the detected sensitivity and specificity of the traditional serum tumor markers, such as carcinoembryonic antigen (CEA), carbohydrate antigen 15-3 and 27.29 (CA15-3, CA27.29), were low and should be used along with the physical examination and diagnostic imaging tests.In addition, tumor markers are expressed heterogeneously in breast cancer and the detection of single marker may gain unsatisfactory specificity.A panel of mRNAwas suggested to potentiallyimprove accuracy and reliability[3].Therefore, this study aims to detect circulating tumor cells (CTCs) in breast cancer using a multiple-marker assay developed in our laboratory previously[4]. Hopefully, the multi-marker assay becomes an effective tool for breast cancer detection and follow-up.

Materials and Methods

Specimen collections

Thirty paired tumor tissues and 87 peripheral blood samples were collected from patients with breast cancer at Kaohsiung Medical University Hospital. Besides, peripheral blood samples taken from 64 healthy volunteers served as controls. All specimen donors were females, and the age distribution of volunteers and patients

were similar.

Microarray and bioinformatics analysis

Three pairs of normal and breast cancer tissues in different cell type were selected for two-color microarray experiments by using Agilent Human 1A (V2) Oligo chip (Agilent Technologies Inc., Santa Clara,USA)according to the manufacturer's instructions. The data of microarray were analyzed by using GeneSpring software (Agilent Technologies). Twenty genes were selected since their expression level were significantly higher in breast cancer tissue than in normal tissue ($p<0.05$).

Total RNA Extraction and cDNA Synthesis

Total RNA was isolated from tissue and blood specimens of patients and control by usingRNeasy Mini Kitand QIAamp RNA Blood Mini Kit (QIAGEN Inc., Valencia, USA)respectively. First strand cDNA was synthesized from total RNA by using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics Ltd., Germany).

Oligonucleotide membrane array

The oligonucleotide probe sequences of20 candidate genes were shown in Table 1.The procedure of membrane array was performed according to our published methods[4]. The density of each gene was divided with β-actin as an internal control. In tissue sample validation, the expression ratio of each gene was calculated by the quantification thatthe spot densities of tumor tissue divided with paired normal tissue. The gene was taken as over-expressed when the ratio was more than two. In blood sample test, each gene was considered as positive according to the cut-off values.

Statistical analysis

All data were analyzed using the Statistical Package for the Social Sciences Version 12.0 software (SPSS Inc., Chicago, USA). Receiver-operating characteristic (ROC) curves were constructed by plotting all possible sensitivity/specificity pairs for each gene and the membrane arrays analysis, resulting from continuously varying the

cut-off values over the entire range of results obtained.

Results

Tissue samples and blood sampleswere used for gene expression confirmation by membrane array. While the mean spot densities consistently varying by a factor more than two were taken as positive, the positive rate of each gene in 30 pairs of normal and breast cancer tissue were shown in Table 2. Nineteen genes had more than 2-fold over-expression in tumortissues compared with those in normal tissues in 80% paired samples. Then, gene expression in the peripheral blood samples from 87 breast cancer patients with every stage and 64 normal volunteers were analyzed using membrane array. The densities were calculated by ROC analysis, and each gene got a total area under the curve (AUC) value. According to the AUC value of each gene, 8 genes were selected as the diagnostic markers since their AUC values were more than 0.8.The cut-off-value of each gene on considering as positive was in accordance to the optimal sensitivity and specificity. Furthermore, 8 genes were combined for ROC analysis and the cut-off point was set as 5 to read the chip positive. At this cut-off point, the sensitivity and specificity of the 8 markers in detecting circulating cancer cells is 90% and 89%, respectively (Figure 1).

Discussion

Among 20 genes selected from microarray analysis, most genes have been reported to be correlated with breast tumor in several studies[5, 6]. A clinical validation by the combination of multiple markers was performed in the present study. Compared to that of other multiple marker assay[7, 8], the detected sensitivity and specificity of our membrane array was effectively elevated to approximately 90%. This membrane array was demonstrated to be a highly sensitive and reliable method for detecting CTCs in the peripheral blood of breast cancer patients. In addition, it might be useful to apply

for postoperative follow-ups because of its convenience. To improve early diagnosis or prognosis of breast cancer, the correlations between the gene expression patters in membranearray and clinical pathological phenotype of breast cancer patients need to be studied further.

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Figure 1. Result of ROC curve analysis for combining 8 selected genes. When the cut-off point was set as 5 to read the membrane array positive, the detected sensitivity and specificity of the 8 markers on circulating cancer cells is 90% and 89% respectively.