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Gene expression profile in breast cancer comprising predictive markers for metastatic risk

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ABSTRACT. Quantitative multiplex reverse transcriptase-polymerase chain reaction was developed for the simultaneous detection of multiple-gene expression levels of formalin-fixed, paraffin-embedded breast cancer samples. Candidate genes were selected from previous microarray data relevant to breast cancer markers that had the potential to serve as predictive markers for metastatic risk. This multiplex gene set included 11 candidate and 3 housekeeping genes, and the aim was to predict breast cancer progression based on lymph node involvement status. Our study demonstrated that the system generated a good standard curve fit ($R^2 = 0.9901$-0.9998) correlated with RNA concentration. The multiplex gene expression profile indicated significantly downregulated levels of G protein-coupled receptor kinase interacting ArfGAP 2 (*GIT2*) and mitochondrial transcription termination factor (*MTERF*)

genes in a lymph node-positive group of patients, with P values of 0.004 and 0.038, respectively. Therefore, this in-house method using multiple genes of interest might be an alternative tool for prediction of breast cancer metastasis.

Key words: Multiplex RT-PCR; Breast cancer; Metastasis; *GIT2*; *MTERF*

INTRODUCTION

Distant metastasis is the most frequent type of breast cancer recurrence, which not only associates with unfavorable prognosis, but also appears to be a cause of death within 5 years compared with loco-regional events. The risk of distant metastasis is associated with tumor size, axillary lymph node involvement, lymphovascular invasion, hormone receptor status, and human epidermal growth factor receptor-2 status (Saphner et al., 1996; Jatoi et al., 1999; Rugo, 2008; Hernandez-Aya et al., 2011). Axillary lymph node involvement is normally determined by examination under a microscope for the presence of cancer cells. If the axillary lymph nodes, which are the first to receive lymphatic flow, are infiltrated with cancer cells, there will be a greater chance that cancer cells have spread to distant site(s). Hence, successful breast cancer screening could be helpful for cancer staging and treatment selection to decrease the mortality rate of this disease.

The GenomeLab GeXP Genetic Analysis System is a multiplexed reverse transcriptase-polymerase chain reaction (RT-PCR) strategy licensed to Beckman Coulter (Brea, CA, USA) that combines the use of gene-specific and universal primers to maintain the gene ratio in RNA samples during the PCR amplification. Recently, various studies have defined sensitivity and specificity for this novel method. They include: a gene signature for prostate cancer (Rai et al., 2009); serotyping of 9 enteroviruses associated with hand, foot, and mouth disease (Hu et al., 2012); an inflammatory gene expression profile for colon cancer (Drew et al., 2011; Farquharson et al., 2012); identification of human respiratory types/subtypes using 16 targeted sequences (Li et al., 2012); 11 human papillomavirus genotypes (Yang et al., 2012); an immunoregulation gene set related to allograft rejection (cardiac graft) (Xie et al., 2012); and a gene set to distinguish pandemic influenza A H1N1 from seasonal flu (Qin et al., 2010). The method is rapid, cost-effective, has a high throughput, and can be used as an alternative to conventional technologies. Moreover, the GeXP system is suitable for the follow-up and validation of the gene set from microarray experiments.

In this study, the data we obtained from the quantitative multiplex RT-PCR approach demonstrated significantly downregulated expression of G protein-coupled receptor kinase interacting ArfGAP 2 (*GIT2*) and mitochondrial transcription termination factor (*MTERF*) genes in a lymph node-positive group of patients, thereby suggesting that this alternative method might be applied to risk assessment of breast cancer metastasis.

MATERIAL AND METHODS

Total RNA extraction

This study was approved by the Institutional Review Board (IRB), Faculty of

Medicine, Chulalongkorn University (COA No. 111/2012). Formalin-fixed, paraffin-embedded (FFPE) tissues from breast cancer patients were obtained from King Chulalongkorn Memorial Hospital in 2010. FFPE specimens were collected from 20 anonymous patients who were subjected to biopsy for histological examinations. The specimens were classified into 2 groups according to the microscopic findings of a pathologist at King Chulalongkorn Memorial Hospital. The group of specimens from breast cancer patients who had a negative result for axillary lymph nodes disclosing neoplastic cell infiltrates was named the "lymph node-negative group", while the group with positive results was named the "lymph node-positive group". Two consecutive 10-µm sections were collected from each tissue block, and total RNA isolation was then carried out using an SPRI-TE FFPE NA extraction kit (Beckman Coulter) according to manufacturer instructions with minimal adjustments (Sirirattanakul et al., 2012). Quantitation of total RNA was performed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

Custom design of multiple-gene targets

For the multiplex gene panel, each gene was selected based on previous microarray data and their attributes in association with breast cancer, as well as their potential to serve as predictive gene markers of metastasis (van't Veer et al., 2002; Wang et al., 2005; Feng et al., 2007; Karlsson et al., 2008). The GenomeLabeXpress Profiler software (Beckman Coulter) and the primer-BLAST program (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) were employed to design specific primers for a multiplex gene panel that included 11 targeted genes and 3 housekeeping genes. The 11 targeted genes were: gamma-glutamyl hydrolase (conjugase, folylpolygammaglutamyl hydrolase, *GGH*); *GIT2*; adenosine deaminase (*ADA*); selectin L (*SELL*); *MTERF*; cyclin B1 interacting protein 1, E3 ubiquitin protein ligase (*CCNB1IP1*); tumor necrosis factor, alpha-induced protein 3 (*TNFAIP3*); RAB23, a member of the *RAS* oncogene family (*RAB23*); G protein-coupled receptor 183 (*GPR183*); regulator of G-protein signaling 1 (*RGS1*); and pirin (iron-binding nuclear protein, *PIR*). The 3 housekeeping genes were: beta-2-microglobulin (*B2M*) (Sirirattanakul et al., 2012); glucuronidase, beta (*GUSB*); and phosphoglycerate kinase 1 (*PGK1*). The gene-specific primers were designed as intron-spanning primers without known single nucleotide polymorphisms, and the primer of each gene was designed with a 3'-end of the gene-specific sequence and a 5'-end of the universal tag sequence. The amplification products were fragments with sizes ranging from 110 to 193 bp with at least 4 bp in size difference, as shown in Table 1. Singlet tests were performed for all of the primer pairs to ensure that only specified amplicons were detected.

Multiplex RT-PCR analysis

The transcriptional level of each gene was determined using multiplex RT-PCR. The multiplex PCR was operated using a GenomeLab GeXP start kit (Beckman Coulter) with Thermo-StartTaqDNA polymerase (Thermo Fisher Scientific) according to the protocol provided. The amplified products were separated by capillary electrophoresis based on the GenomeLab GeXP Genetic Analysis System. The data were analyzed and normalized against a reference gene and housekeeping genes using the system software. The results were exported for further analysis in a tab-delimited format file.

Table 1. List of primers used for the 14-gene multiplex panel.

Accession No.	Gene symbol	Product length	Forward primer (5'-3')**	Reverse primer (5'-3')**
NM_003878	*GGH*	110	AGGTGACACTATAGAATAAAA TACAGATGGCAAGATTGAGTTT	GTACGACTCACTATAGGGACCA CTGGACACCATATACTGGA
NM_014776	*GIT2*	115	AGGTGACACTATAGAATACTAG CCTTCTATCTCTGTGGCAG	GTACGACTCACTATAGGGAGCT GTCTGCCATTTGAGGTATTATA
NM_004048	*B2M**	120	AGGTGACACTATAGAATACAG CCCAAGATAGTTAAGTGGGA	GTACGACTCACTATAGGGAAAT TCATCCAATCCAAATGCG
NM_000022	*ADA*	125	AGGTGACACTATAGAATATTAC CAGATGACCAAACGGG	GTACGACTCACTATAGGGATGG GAGGAAACTAGATTTGGC
NM_000655	*SELL*	129	AGGTGACACTATAGAATACCTG CCACAAACTAAAGGCA	GTACGACTCACTATAGGGAATT GATGATTTCTACACATTCTCCA
NM_006980	*MTERF*	133	AGGTGACACTATAGAATATAGC TGTTCTCCAGCCTTTCTG	GTACGACTCACTATAGGGATGG TGCCATAATGGTTAGGTAGT
NM_000181	*GUSB**	142	AGGTGACACTATAGAATATGTA CTTCTGTACACGACACCCA	GTACGACTCACTATAGGGACCC TTGACAGAGATCTGGTAATT
NM_182849	*CCNB1IP1*	147	AGGTGACACTATAGAATATGA GTACTGCGTTTTTCTTTTATG	GTACGACTCACTATAGGGATGC GACACTTTCGATAATTACAA
NM_006290	*TNFAIP3*	158	AGGTGACACTATAGAATAAGG AATGCTACAGATACCCCA	GTACGACTCACTATAGGGACCC CGGTCTCTGTTAACAAG
NM_183227	*RAB23*	163	AGGTGACACTATAGAATAATG ATTCAGCGATATTGCAAA	GTACGACTCACTATAGGGATGC AGTGTCCCATAACATTAGTC
NM_004951	*GPR183*	168	AGGTGACACTATAGAATATACA CAGAGACCCGAACGAGT	GTACGACTCACTATAGGGACTG TGATGTGCATAGAGGTCAC
NM_000291	*PGK1**	176	AGGTGACACTATAGAATATTGA CCGAATCACCGACC	GTACGACTCACTATAGGGAGGT TGTTCTTCATAGGAACATTGA
NM_002922	*RGS1*	180	AGGTGACACTATAGAATATGGC TGGCTTGTGAAGACTAT	GTACGACTCACTATAGGGATTC TTGGCTGTAGATTCTCGAG
NM_003662	*PIR*	193	AGGTGACACTATAGAATAGGG GAGCCATTAAGAGAACC	GTACGACTCACTATAGGGACGC TTTCCACTAGTTCCCAA

*Housekeeping gene used for the multiplex panel. **Universal tag sequences are underlined.

Statistical analysis

The results of normalized gene expression ratios for all genes of interest were analyzed by the Student *t*-test using Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA). The overall correlations among selected genes and clinical outcomes were calculated through Pearson's correlation analysis by the SPSS software, version 17.0 (IBM Corporation, Armonk, NY, USA).

RESULTS

Individual primer pair testing

The singlet tests with the multiplex of reverse primers against an individual forward primer showed that all genes were successfully amplified at the expected sizes with a peak at 325 nucleotides for the kanamycin-resistance (*KANr*) internal control. All of the expected fragments and the size standards were observed as the blue and red peaks, respectively. The results are illustrated in Figure 1.

Gene expression profile analysis

In this study, the multiplex gene set containing 11 candidate genes (*GGH*, *GIT2*, *ADA*, *SELL*, *MTERF*, *CCNB1IP1*, *TNFAIP3*, *RAB23*, *GPR183*, *RGS1*, and *PIR*), 3 housekeeping

genes (*B2M*, *GUSB*, and *PGK1*), and *KANr* (a reference RNA) was successfully generated and resulted in product sizes ranging from 110 to 193 bp. This multiplex was found to be suitable for breast cancer detection. After a standard curve for the multiplex was created, the linearity was detected for concentrations ranging from 15.5 to 1000 ng in every gene (R^2 = 0.9901-0.9998).

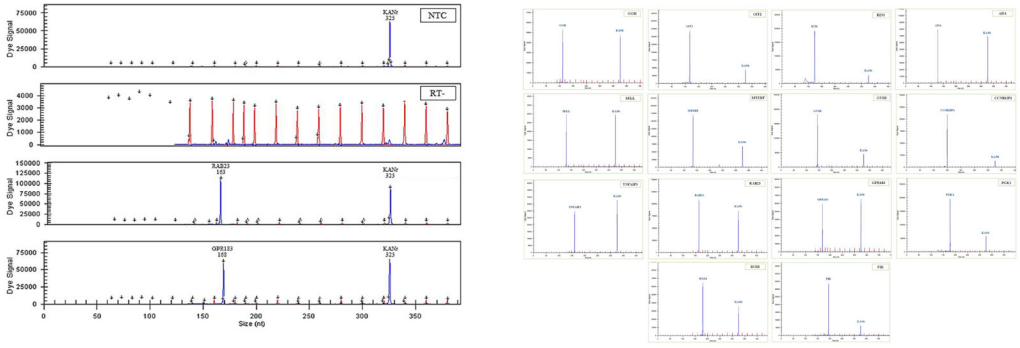

Figure 1. Singlet reaction results are demonstrated for **A.** no template control (NTC), RT-minus control (RT-), *RAB23*, and *GPR183*. **B.** Results of singlet reactions of other genes. All of the expected fragments were observed as the blue peaks at 110, 115, 120, 125, 129, 133, 142, 147, 158, 163, 168, 176, 180, 193, and 325 nucleotides (nt) for *GGH*, *GIT2*, *B2M*, *ADA*, *SELL*, *MTERF*, *GUSB*, *CCNB1IP1*, *TNFAIP3*, *RAB23*, *GPR183*, *PGK1*, *RGS1*, *PIR*, and *KANr* (reference primer), respectively. The red peaks represent the size standards.

The expression profile of the 11 candidate genes was assessed for comparison between the lymph node-negative and lymph node-positive breast cancer groups simultaneously. Significant differential expression was observed for 2 genes, *GIT2* (P = 0.004) and *MTERF* (P = 0.038). These 2 genes were significantly downregulated in lymph node-positive breast cancer with 1.94- and 2.22-fold changes, respectively (Figure 2). For the remaining genes, no significant differential expression was observed, although the upregulation of *PIR* and *CCNB1IP1* genes was seen in the lymph node-positive group with 1.81- and 2.1-fold changes, respectively (P > 0.05).

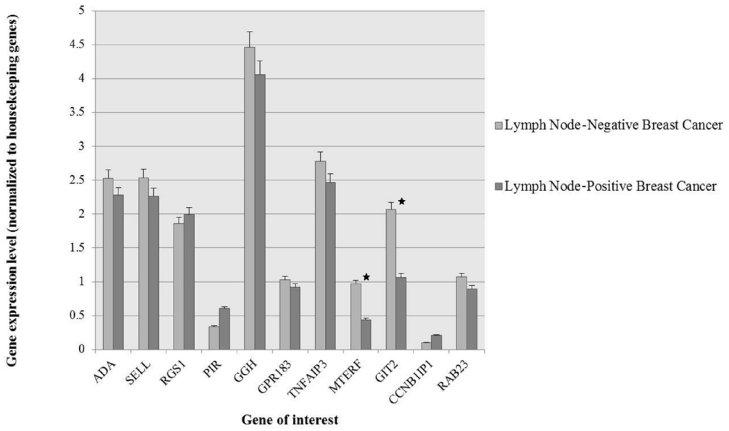

Figure 2. Normalized expression ratio of each gene against housekeeping genes is shown on the primary vertical axis comparing lymph node-negative breast cancer and lymph node-positive breast cancer. Differential expression of *GIT2* and *MTERF* genes was found to be significant with P values of 0.004 and 0.038, respectively.

The overall correlation of gene expression and the correlation among gene expression levels and clinical data were analyzed using Pearson's correlation analysis. Significant correlation at a level of $P < 0.01$ was found in *SELL-ADA-TNFAIP3*, *SELL-RGS1*, *CCNB1IP1-PIR*, and *PIR-MTERF*. Significant correlation at $P < 0.05$ was found in *SELL-GIT2*, *SELL-CCNB1IP1*, *CCNB1IP1-RGS1*, *RGS1-TNFAIP3*, *RGS1-GPR183*, *GPR183-CCNB1IP1*, and *GPR183-PIR*. According to the correlation among gene expression levels and clinical data, estrogen receptor-positive percentage showed an inverse correlation to *SELL*, *MTERF*, and *CCNB1IP1* at significance levels of 0.004, 0.024, and 0.003, respectively, while progesterone receptor-positive percentage showed a significant correlation to the Ki-67-positive percentage at a P level of 0.024.

DISCUSSION

The breast cancer multiplex primer set containing 11 candidate genes, 3 housekeeping genes, and *KANr* was successfully generated and found to be suitable for breast cancer detection. Two of the 11 genes, *GIT2* and *MTERF*, could be used to distinguish lymph node-negative from lymph node-positive breast cancer patients. The changing tendency of expression was also observed in other genes, but with no statistical significance. This lack of significance might be due to sample size limitations. Gene expression correlations were found in this study along with co-expression patterns analyzed by the computation-based method (GeneMANIA; http://www.genemania.org) together with GEO data (data not shown).

The role of *GIT2* and *MTERF* in cancer regulation was investigated. *GIT2* knockdown increased tumor cell invasion and migration, and was also involved in focal adhesion turnover, membrane protrusion extension, and the level of CT10 regulator of kinase adaptor protein, which has been shown to play key roles in cell motility (Frank et al., 2006; Fathers et al., 2012; Tomar et al., 2012). Moreover, *GIT2* regulation through the GIT2-PIX-PAK1-G$\beta\gamma$ complex is involved in superoxide production and immunological synapse regulation, which are also considered to be hallmarks of cancer (Premont et al., 2000; Mazaki et al., 2006; Sabe et al., 2006; Hanahan and Weinberg, 2011). It is worth noting that loss of *MTERF* has been reported to cause a respiratory defect (Wenz et al., 2009). The mitochondrial respiration defect induces pseudo-hypoxic pathway-mediated hypoxia-inducible factor, which could be a tumor development regulator that enhances tumor cells' ability to spread and survive through neovascularization and anti-apoptotic facilitation (Gottlieb and Tomlinson, 2005). However, further studies are needed to delineate the gene function and its possible relevance to cancer progression regulation. A study on a larger population concerning the correlation of these gene expression signatures corresponding to survival time would be key to assessing the value of using these possible prognostic markers.

The GeXP Genetic Analysis System, a multiplex end-point RT-PCR amplification-based method incorporating a dye-labeled capillary electrophoresis detection system, served as an alternative technique to the RNA-based applications. The GeXP system is considered to be rapid, cost-effective, and to have a high throughput (Rai et al., 2009; Drew et al., 2011). The sensitivity and specificity of the system were found to be comparable to conventional and other methods (Qin et al., 2010; Hu et al., 2012; Li et al., 2012; Xie et al., 2012). However, certain limitations of the GeXP system were noted. First, the analyzed gene set must be appropriate for the detection range, and each fragment should be at least 5 bp apart. Regarding the FFPE experiment, RNA is normally fragmented resulting in a limited target range of 100-200

bp (including the universal tag sequences), and the maximum number of primers that can be generated is 20 primer pairs. Second, an optimization step is needed for each primer to obtain the optimal signal for detection. Third, mistakes can occur during the multiple steps of the system, such as RNA extraction, RT, and PCR steps. A standard procedure to lessen the chance of error would be helpful.

In conclusion, our quantitative multiplex RT-PCR approach showed significantly downregulated expression of the *GIT2* and *MTERF* genes in FFPE samples of the lymph node-positive group of breast cancer patients, thereby suggesting that this alternative technique might be useful for risk assessment of breast cancer metastasis.

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Conflicts of interest

The authors declare no conflict of interest.

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