

Original Article

Determination of whole transcription profiles and specific pathways in invasive ductal breast carcinoma

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Abstract: Breast cancer is the most common cancer affecting women worldwide including Thailand. Whole transcription profiles of invasive ductal breast carcinoma (IDC) obtained by oligonucleotide microarray should lead to a better understanding of the molecular basis of IDCs, allow for examination of specific markers for diagnosis, and provide novel targets for therapy. This study aimed to detect the whole transcript expression of approximately 35,000 target genes in Thai breast cancer patients, using Affymetrix GeneChip[®] Exon 1.0 Sense Target Arrays. Analysis revealed that the differential expression profiles of 928 genes (423 up-regulated and 505 down-regulated genes) were 2-fold or greater (unpaired t-test, $p < 0.05$) in invasive ductal breast cancer, compared with normal tissues. The Gene Ontology (GO) databases support important associations in 17 gene sets with p -value $< 1E-10$ and ≥ 4 -fold changes, involving the tumorigenic pathways of cell cycles, extracellular regions, as well as cellular component organization. Likewise, the TGFBR and IL-6 pathways contain gene expression with statistically significant changes in IDC.

Keywords: Invasive ductal breast carcinoma, oligonucleotide microarray, gene expression profile, TGFBR, IL-6 pathway

Introduction

Breast cancer is the most common cancer affecting women worldwide, and in Thailand [1]. It is a complex and heterogeneous disease with varied morphological manifestations, molecular features, behaviors, and responses to therapy. In Thailand, 80% of the estimated incidence rate of breast cancer is invasive ductal breast carcinomas (IDC) [2]. These types of tumor commonly metastasize to the axillary lymph nodes, and their prognosis is often more severe than other special histological types [3]. Early diagnosis of breast cancers, especially IDC types, can offer patients a wider range of therapeutic options, greater therapeutic success, and lower rates of mortality. Molecular methods provide prognostic and predictive information, and can help to identify new thera-

peutic targets and molecular classifiers. At present, the main large-scale application of microarrays is comparative expression analysis. Microarray technology makes it possible to analyze the expression profiles of thousands of genes in parallel [4-6], and breast-cancer research is one of the most advanced fields of microarray research. These cancers may be classified into several subtypes based on the unique gene expression pattern of each cancer-cell specimen. It is also possible to use the results in diagnosis and in the prediction of therapeutic sensitivity [7-9]. This study aimed to detect the whole transcript expression of approximately 35,000 target genes in Thai breast-cancer patients, using Affymetrix GeneChip[®] Exon 1.0 Sense Target Arrays. The results may provide banking data for exon-level expression profiles underlying IDC, and indicate

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Table 1. Clinicopathological features of the 19 invasive ductal breast carcinoma samples

Clinical Characteristic	
Age, years (median, range)	54.7, 30-83
Tumor location (Right: Left)	12: 7
Lymph node metastasis (n_0 : $n_{\geq 1}$)	10: 9
TNM stage (stage I+II: III+IV)	13: 6
Prognostic marker (*TNP: non-TNP)	5: 14

*TNP: Triple-negative breast cancer is distinguished by negative immunohistochemistry assays for expression of ER, PR and HER2.

the locations of novel genetic targets and specific pathways, which may prove useful for IDC diagnostic markers and therapy.

Materials and methods

Clinical specimen collection

Nineteen (19) cases of IDC and 7 normal breast tissues were obtained from the Pathology Division of the Army Institute of Pathology, Bangkok, Thailand, during surgical resectioning. The median age at diagnosis was 54.7 years (range 30-83). This work was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Thailand (MUTM 2008-004-04). After resectioning, all specimens were immediately stored in TRIzol® (Invitrogen, CA, USA) at -80°C prior to use. None of the patients underwent pre-surgical radiation or chemotherapy. Hematoxylin and eosin-stained sections and immunohistochemically stained fixed tissues were examined microscopically by a pathologist. The patients' clinical and histopathological characteristics are shown in **Table 1**.

RNA preparation

Total RNA was isolated from breast tissue with TRIzol® according to the manufacturer's instructions. All RNA samples were assayed for standard quality to ensure that only the highest quality RNA would be hybridized for gene expression arrays. The quantity of RNA was measured by reading absorbance at 260 nm and 280 nm by NanoDrop™ 1000 (Thermo Fisher Scientific, MA, USA). Acceptable A260/280 ratios were in the range 1.8-2.1. RNA quality was analyzed by agarose gel electrophoresis, with the 28S rRNA band appearing approximately twice as intense as the 18S

rRNA band. For quantitative RT-PCR, 2.5 µg total RNA was reverse-transcribed with a SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, CA, USA) by thermal cycler (GeneAmp 9700, Perkin-Elmer, USA). The *ACTB* (β -actin), a housekeeping gene, served as a control gene to check the consistency of the reverse transcription.

Whole-transcript expression array and microarray image processing

One µg/µl of high quality total RNA was used as a starting material for making total RNA/Poly-A RNA controls, and was mixed using a GeneChip® Eukaryotic Poly-A Control Kit (Affymetrix, Inc., CA, USA). The majority of the rRNA was removed from the total RNA samples prior to target labeling, so as to increase sensitivity by RiboMinus™ Human/Mouse Transcriptome Isolation Kit (Invitrogen, CA, USA); cDNA was synthesized using the GeneChip® WT (Whole Transcript) Sense Target Labeling and Control Reagents Kit, as per the manufacturer's instructions (Affymetrix, Inc., CA, USA). The sense cDNA was then fragmented by UDG (uracil DNA glycosylase) and APE 1 (apurinic/apyrimidinic endonuclease 1), and biotin-labeled with TdT (terminal deoxynucleotidyl transferase) using a GeneChip® WT Terminal Labeling Kit (Affymetrix, Inc., CA, USA). After the biotin-labeled sense target DNA was prepared, the sample was ready to hybridize to gene chip (The GeneChip® Human Exon 1.0 ST array). Hybridization was performed using 5 µg of biotinylated target, which was incubated with a GeneChip® Hybridization, Wash and Stain Kit and a GeneChip® Fluidics Station 450 (Affymetrix, Inc., CA, USA). The arrays were scanned using a GeneChip® Scanner 3000 7G (Affymetrix, Inc., CA, USA). Raw data were extracted from the scanned images and analyzed with GeneSpring GX software version 11.5 (Agilent Technologies, CA, USA).

Data analysis

Microarray data were analyzed by GeneSpring GX software version 11.5 (Agilent Technologies, CA, USA). The data were normalized using the iterative PLIER default protocol. Significant differentially expressed genes were analyzed by unpaired t-test. Benjamini-Hochberg false discovery rate multiple test correction was conducted, where applicable. All statistically differ-

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Table 2. Primer sequences of the selected genes for quantitative RT-PCR

Gene Symbol	Gene description	Primer sequences	Product size (bp)
<i>ACTB</i>	β -Actin	Forward: TCACCCACACTGTGCCCATCTACGA Reverse: CAGCGGAACCGCTCATTGCCAATGG	294
<i>ANLN</i>	Anillin, actin binding protein	Forward: TCACTGGAAGCCGAGAGGAGAGGA Reverse: GCGTCGGACTACGAGACGATGGAA	150
<i>CCNB1</i>	Cyclin B1	Forward: AGGAAGAGCAAGCAGTCAGACCA Reverse: TGCAGCATCTTCTTGGGCACACAA	190
<i>COL10A1</i>	Collagen, type X, alpha 1	Forward: AGAATATGCTGCCACAATACC Reverse: CCTCTACTGCTATACCTTACTC	175
<i>FABP4</i>	Fatty acid binding protein 4, adipocyte	Forward: AAAGTCAAGAGCACCATAACCTT Reverse: TCTCACCACCAGTTTATCATCCT	114
<i>INHBA</i>	Inhibin, beta A	Forward: GGATGCCCTTGCTTTGGCTGAGAG Reverse: TCTTGACGGCCTCCACCATCTCTG	180
<i>LYVE-1</i>	Lymphatic vessel endothelial hyaluronan receptor 1	Forward: TTGAAACAGCCTTAAAAGCTA Reverse: GAGTTAGTCCAAGTATCAGATGA	193
<i>SQLE</i>	Squalene epoxidase	Forward: CCAGTGCCGAGGTGTTTCTGTGAC Reverse: ACCAAGAGCAGGTGCCCTTTCAGA	70
<i>TNS1</i>	Tensin 1	Forward: TCAAGTGAAGAACTTGTGTTGCTT Reverse: CACGACAATATAGTGGAGGCACA	86
<i>TOP2A</i>	Topoisomerase (DNA) II alpha	Forward: CTGGACCAACCTTCAACTATCTTC Reverse: CTTGGCTTCAACAGCCTCCAATTC	191
<i>TPX2</i>	Microtubule-associated, homolog (<i>Xenopus laevis</i>)	Forward: GCTAATAACGGTTCTTGATACATA Reverse: CCACTTAACGCAGAAGAGCAG	181

ent genes (p -value < 0.05) with a greater than 2-fold increase or decrease were accepted for generation. Hierarchical cluster analysis was used to assess correlations among samples for each identified gene set with Euclidean distance and average linkage statistical methods.

Confirmation of microarray data by quantitative RT-PCR

To confirm the results obtained by microarray, 10 genes were selected randomly for differential expression from the gene list with > 2-fold increase or decrease in quantitative RT-PCR analysis. The set of primers were designed from the known sequence obtained in GenBank, using sequence analysis as well as Primer-BLAST, an interactive web-based program for designing degenerate primers (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). The primer sequence of these genes is shown in **Table 2**. The *ACTB* gene in the tumors and normal tissue was also quantified as the control gene copy number. Gene expression was quantified by quantitative

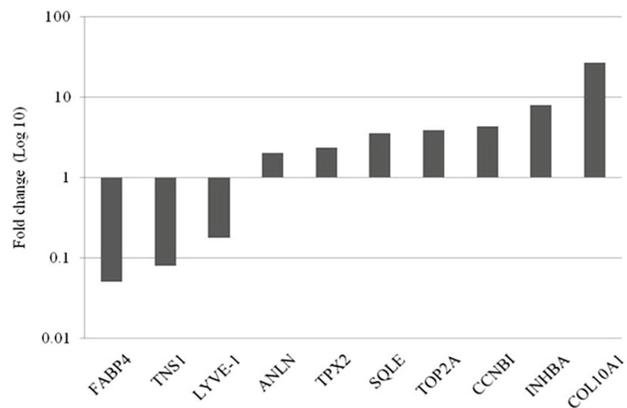


Figure 1. Determination of gene expression profiles of selected genes. The gene expression of *FABP4*, *LYVE-1*, *TNS1*, *TOP2A*, *TPX2*, *INHBA*, *ANLN*, *SQLE*, *CCNB1*, and *COL10A1* were analyzed by real-time RT-PCR. The *ACTB* gene in the tumors and normal tissue was quantified as the control gene copy number.

RT-PCR using the SYBR Green I PCR Kit (Roche Diagnostics, Germany). Quantitative RT-PCR was performed in a LightCycler® 1.0 Instrument (Roche Diagnostics, Germany). Thermal cycling and fluorescent monitoring were performed. The point at which the PCR product was first detected above the fixed threshold, termed the cycle threshold (Cp), was determined for each

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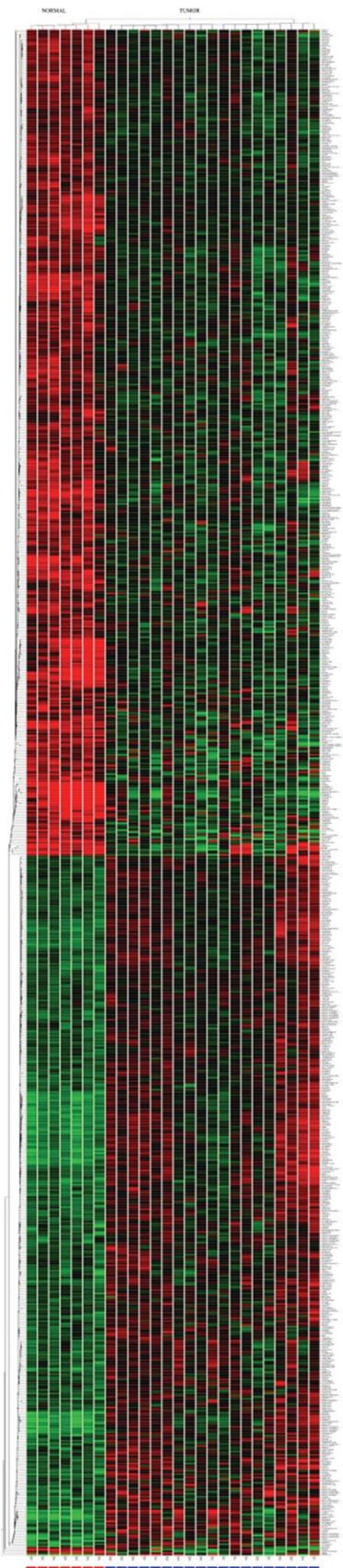


Figure 2. Hierarchical cluster analysis of 19 IDC and 7 normal samples, according to 928 differentially expressed genes (p -value < 0.05 with ≥ 2 -fold change). Columns represent samples; rows represent individual probes. The heat map depicts high (red) and low (green) relative levels of gene expression.

sample. Relative gene amplifications in breast tissue DNA were determined by comparative Cp, as described by Livak *et al* [10].

Results

Confirmation of microarray data by quantitative RT-PCR

Ten (10) genes, randomly selected from the list of 928 genes – *FABP4* (fatty acid binding protein 4, adipocyte), *LYVE-1* (lymphatic vessel endothelial hyaluronan receptor 1), *TNS1* (tensin 1), *TOP2A* (topoisomerase (DNA) II alpha), *TPX2* (microtubule-associated, homolog (*Xenopus laevis*)), *INHBA* (inhibin, beta A), *ANLN* (anillin, actin binding protein), *SQLE* (squalene epoxidase), *CCNB1* (cyclin B1), and *COL10A1* (collagen, type X, alpha 1) – were subjected to quantitative RT-PCR (**Figure 1**). The internal control gene *ACTB* was used in a normalization procedure. Using the initial sample sets and the criteria of ≥ 2 -fold change, 100% yielded consistent results using the two technologies.

Gene expression profile of invasive ductal breast carcinoma

Changes in gene expression in IDC were analyzed and compared with normal controls. Differential expression in IDC was noted in 928 genes at p -value < 0.05 with a ≥ 2 -fold change. Of these, 423 were up-regulated and 505 down-regulated. The largest change among the down-regulated genes was > 22 -fold decrease in *FABP4* (fatty acid binding protein 4, adipocyte), while among the up-regulated genes, the largest change was a 9-fold increase in *COL10A1* (collagen, type X, alpha 1). A hierarchical cluster analysis of all samples with p -value < 0.05 and an ≥ 2 -fold change is shown in **Figure 2**. Using the GO databases functional annotation to describe the biological functions of the gene sets that were associated with IDC with p -value $< 1E-10$ and an ≥ 4 -fold change, the 17 gene sets revealed that they are involved in cell cycles and cell cycle processes such as M phase, nuclear division, organelle fission, extracellular regions and cellular component organization

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Table 3. Gene ontology analysis of intraductal breast cancer with different biological processes ($p < 0.05$, -fold change ≥ 4)

GO ACCESSION	Functional Name	Genes in Detection	Genes in Total	Corrected p -value
GO: 0000087	M phase of mitotic cell cycle	33/30	274	1.05E-19
GO: 0000278	mitotic cell cycle	37/34	432	3.68E-18
GO: 0000279	M phase	37/34	402	1.06E-18
GO: 0000280	nuclear division	31/29	266	1.50E-18
GO: 0006996	organelle organization	57/44	1605	1.39E-11
GO: 0007049	cell cycle	44/41	929	1.83E-12
GO: 0007067	mitosis	31/29	266	1.50E-18
GO: 0007346	regulation of mitotic cell cycle	21/18	187	8.25E-12
GO: 0010564	regulation of cell cycle process	24/21	223	2.85E-13
GO: 0016043	cellular component organization	78/64	2700	3.56E-12
GO: 0022402	cell cycle process	40/37	660	1.29E-14
GO: 0022403	cell cycle phase	40/37	496	1.49E-18
GO: 0044421	extracellular region part	50/43	1048	1.29E-14
GO: 0048285	organelle fission	31/29	275	3.41E-18
GO: 0051301	cell division	32/30	359	6.95E-16
GO: 0071840	cellular component organization or biogenesis	78/64	2855	5.91E-11
GO: 0071842	cellular component organization at cellular level	68/54	2243	3.93E-11

(Table 3). Genes up-regulated were enriched for the GO term cell cycles and cell cycle processes, while genes down-regulated were enriched for the extracellular region part.

Significant pathway analysis

Significant pathway analysis revealed that several gene alterations in TGFBR (transforming growth factor, beta receptor) and IL6 (interleukin 6) pathways had statistically significant differential expression in IDC. For the TGFBR pathway, there were significant changes in the levels of gene expression in 11 of 145 genes (Table 4), whereas significant changes in gene expression in 5 of 48 genes in the IL6 pathway were observed (Table 5).

Discussion

Recently, the microarray-designed technology of the Exon array has been improved with its greater number of probe sets as part of the new database, and results in high resolution. In this study, the gene expression profiles of IDC in Thai patients were analyzed using oligonucleotide microarray. By means of unsupervised hierarchical clustering algorithms and random permutation tests, different expression profiles were revealed between normal and breast can-

cer tissue samples. Using a cut-off point of p -value < 0.05 with > 2 -fold change, 928 genes were found to be differentially expressed. Among these, *COL10A1* and *FABP4* had the most extreme -fold change of the up-and down-regulated genes in IDC tissue, when compared with normal tissue. *COL10A1* encodes the alpha chain of type X collagen as a component of the extracellular matrix, and may be involved in invasion and metastasis of cancer cells [11]; it can also be used as a novel target for the diagnosis and treatment of diverse solid tumor types [12]. Down-regulation of *FABP4* has been associated with obesity [13] and also risk of bladder cancer [14], and is reiterated by the present study. Increasing *FABP4* in plasma has also previously been linked to obese breast cancer patients [15]. It should be noted that the top 20 up- and 20 down-regulated genes presented in this study were similar to previous investigations [16-18]. However, novel changes in gene expression levels of *CHRD1* (chordin-like 1), *GPD1* (glycerol-3-phosphate dehydrogenase 1 (soluble)), *COL17A1* (collagen, type XVII, alpha 1), and *GTSE1* (G-2 and S-phase expressed 1) in IDC were found only in the present study. Among these novel genes, the overexpression of *GPD1* protein was a new candidate biomarker for colon cancer [19].

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Table 4. List of genes in the TGFBR pathway with significant expression in IDC (p -value < 0.05 and ≥ 2 -fold change)

	Reference sequence	Gene symbol	Gene description	Location	Fold change
1	NM_001786 NM_033379 NM_001170406 NM_001170407	<i>CDK1 CDC2</i>	cyclin-dependent kinase 1 cell division cycle 2, G1 to S and G2 to M	nucleus	5.626458
2	NM_004701	<i>CCNB2</i>	cyclin B2	nucleus	4.783654
3	NM_001238	<i>CCNE1</i>	cyclin E1	nucleus	2.6909218
4	NM_002895 NM_183404	<i>RBL1</i>	retinoblastoma-like 1 (p107)	nucleus	2.225321
5	NM_001789 NM_201567	<i>CDC25A</i>	cell division cycle 25 homolog A (S. pombe)	nucleus	2.0046442
6	NM_005354	<i>JUND</i>	jun D proto-oncogene	nucleus	-2.164308
7	NM_001024847 NM_003242	<i>TGFBR2</i>	transforming growth factor, beta receptor II (70/80kDa)	membrane	-2.4941776
8	NM_005252	<i>FOS</i>	FBJ murine osteosarcoma viral oncogene homolog	nucleus	-2.992894
9	NM_001172895 NM_001753 NM_001172896 NM_001172897	<i>CAV1</i>	caveolin 1, caveolae protein, 22kDa	cytoplasm	-4.6306677
10	NM_003243	<i>TGFBR3</i>	transforming growth factor, beta receptor III	membrane	-5.143685
11	NM_006732 NM_001114171	<i>FOSB</i>	FBJ murine osteosarcoma viral oncogene homolog B	nucleus	-6.2435536

Table 5. List of genes in the IL-6 pathway with significant expression in IDC with a p -value < 0.05 and fold change ≥ 2 -fold

	Reference sequence	Gene symbol	Gene description	Location	Fold change
1	NM_004219 NR_002734	<i>PTTG1</i>	pituitary tumor-transforming 1	cytoplasm	3.455604
2	NM_004935 NM_001164410	<i>CDK5</i>	cyclin-dependent kinase 5	cytoplasm	2.476688
3	NM_003955	<i>SOCS3</i>	suppressor of cytokine signaling 3	cytoplasm	-2.389107
4	NM_005252	<i>FOS</i>	FBJ murine osteosarcoma viral oncogene homolog	nucleus	-2.992894
5	NM_000600	<i>IL6</i>	interleukin 6 (interferon, beta 2)	cytoplasm	-3.360038

The GO databases support important associations in 17 gene sets with p -value $< 1E-10$ and ≥ 4 -fold changes, involving the tumorigenic pathways of cell cycles, extracellular regions, as well as cellular component organization. In addition, many genes in the cell cycle were up-regulated in IDC whereas most gene expression in the extracellular region part was down-regulated. Of note, the novel gene expression in IDC was reported in the cell cycle, extracellular region part, and cellular component organization. Indeed, the following were all found in the present study: *NCAPH* (non-SMC condensin I

complex, subunit H), *CDCA3* (cell division cycle associated 3), *FAM83D* (family with sequence similarity 83, member D), *CCL14-CCL15* (chemokine (C-C motif) ligand 14-15), and *KCNIP2* (Kv channel interacting protein 2). The current study showed that overexpression of *CDCA3* might be associated with oral carcinogenesis, by preventing the arrest of cell cycle progression at the G1 phase via decreased expression of the cyclin-dependent kinase inhibitors [20]. Likewise, all known gene expression in IDC – including *MKI67* (antigen identified by monoclonal antibody *Ki-67*), *KIT* (v-kit Hardy-Zuckerman

4 feline sarcoma viral oncogene homolog), and *CDK1/CDC2* (cyclin-dependent kinase 1) – was consistently found in correlation with other studies [20-22].

By specific pathway analysis, the present study indicated 11 of 145 genes in the TGF β R pathway were significantly expressed in IDC. Several pieces of evidence showed numerous genes, including *CDC2*, *CCNB2*, *CDC25A*, *CCNE1*, *FOS*, *JUND*, *CAV1*, *TGFRB2* and *TGFRB3*, play a role in human cancer carcinogenesis, including breast cancer [23-31]. The *RBL1* gene encoded protein is similar in sequence and possibly function to the product of the *RB1* gene (retinoblastoma 1). It is also thought the protein encoded by the *RBL1* gene may also be a tumor suppressor [32]. In the IL-6 pathway, 5 of 48 genes significantly change in terms of level of gene expression in IDC. Among these, *CDK5* amplification was observed in pancreatic cancer [33]. The expression of *PTTG1*, a new oncogene, was involved in several cancers including clear cell renal cell carcinoma, and has been associated with poor patient prognosis [34], endometrial carcinoma [35], hepatoma cellular carcinoma [36], early oral tumorigenesis [37], and may be a new candidate gene in the clinical management of ER-positive breast cancer [38]. *IL6* encodes a cytokine that has a function in inflammation and the maturation of B cells. The functioning of this gene is implicated in a wide variety of inflammation-associated disease states. Elevated expression of *IL-6* has been detected in multiple tumors [39], whereas the over-expression of *SOCS3* is correlated with earlier stages of and better clinical outcomes in breast cancer [40].

In conclusion, this study reported whole transcript expression of target genes in IDC among Thai patients, using Affymetrix GeneChip[®] Exon 1.0ST analysis. The database should lead to a better understanding of the molecular mechanisms of IDC, and it may be possible to develop a novel diagnostic marker and/or method to predict therapeutic sensitivity of this cancer.

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Conflict of interest

The authors declare they have no conflicts of interest.

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