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Award Number: W81XWH-05-1-0317

TITLE: Identification of Tumor Suppressor Genes by Genetic and Epigenetic
Genome-Scanning

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REPORT DATE: April 2008

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE				<i>Form Approved</i> OMB No. 0704-0188	
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1. REPORT DATE 01-04-2008		2. REPORT TYPE Final		3. DATES COVERED 15 Mar 2005– 14 Mar 2008	
4. TITLE AND SUBTITLE Identification of Tumor Suppressor Genes by Genetic and Epigenetic Genome-Scanning				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-05-1-0317	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Fumiichiro Yamamoto Email: fyamamoto@burnham.org				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The Burnham Institute La Jolla, CA 92037				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. Abstract We used systematic multiplex rt-pcr and dna microarray hybridization methods to examine the expression of the genes in the chromosomal regions of 18q21-qter, 8p, and 1p33-pter, which are often decreased in copy number in breast tumors and cell lines derived from breast cancer. We identified dozens of genes in the chromosomal regions whose expression was frequently diminished or lost in breast cancer cell lines that were examined. We confirmed the results by real-time qrt-pcr. We also examined the expression of those genes in the clinical specimens of breast cancer and observed the down-regulation in expression of some of them in the clinical specimens of breast cancer. Those genes included ccbe1, tcf4, np_115536.1, and np_689683.2 in 18q21-qter, and myom2, np_859074, np_001034551, nrg1, phyip (phyhip), q7z2r7, sfrp1, and sox7 in 8p.					
15. SUBJECT TERMS gene expression, copy number changes					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			USAMRMC
			UU	64	19b. TELEPHONE NUMBER (include area code)

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INTRODUCTION

Normal breast epithelial cells undergo both genetic and epigenetic changes in their malignant progression to cancer (1). In that progression, changes in proto-oncogenes and tumor suppressor genes play an important role. Because the (sub-) chromosomal loss (or decrease) of tumor suppressor genes and the gain (or amplification) of proto-oncogenes may confer cells with growth advantages, these changes provide an important tool to identify genes that are important in carcinogenesis. Several consistent changes have been reported, from karyotyping analyses of breast tumors and cancer cell lines (2, 3). By scanning the changes in gene expression and DNA methylation, we proposed to identify tumor suppressor genes in the chromosomal regions of 1p33-pter, 8p, and 18q, three of the regions that are most consistently decreased in copy number.

BODY

The following four tasks and timelines were proposed:

Task 1. To construct oligodeoxynucleotide microarrays representing *NotI* sites in the chromosomal regions of 1p33-pter, 8p, and 18q (months 1–12):

- Identify all the *NotI* sites in chromosomal regions 1p33-pter, 8p, and 18q followed by determining the size of the *NotI*-*MseI* DNA fragments;
- Select, out of roughly 800 *NotI* sites in those regions, more than 500 gene promoter-associated *NotI* sites that are located within 2 kbp from the nearest *MseI* sites;
- Design ~50–60mer oligodeoxynucleotides representing those *NotI*-*MseI* DNA fragments; and
- Prepare oligodeoxynucleotide microarrays.

Task 2. To identify tumor suppressor gene candidates by DNA microarray MS-AFLP (months 9–30):

- Perform the DNA microarray MS-AFLP hybridization experiments using genomic DNA from normal breast epithelial cells and three breast cancer cell lines (MCF7, BT-20, and MDA-MB468); and
- Identify the *NotI* sites that exhibit a decrease in spot intensity in the cells of those three breast cancer cell lines, followed by the examination of neighboring genes.

Task 3. To examine gene expression by SM RT-PCR (months 19–36):

- Establish the SM RT-PCR system composed of ~10 candidate genes located in the 3 chromosomal regions indicated in Task 1;
- Perform SM RT-PCR experiments using RNA from normal breast epithelial cells and three breast carcinoma cell lines (MCF7, BT-20, and MDA-MB468); and
- Identify genes that are down-regulated in expression in those breast cancer cell lines.

Task 4. To examine homozygous deletion by SM PCR (months 19–36):

- Perform the SM PCR experiments using genomic DNA from normal breast epithelial cells and the same three breast carcinoma cell line cells; and
- Identify genes that are homozygously deleted in those breast cancer cell lines.

In Year 1, we planned to perform Task 1 and part of Task 2. We started with Task 1 and identified all the *NotI* sites in the chromosomal regions of 1p33-pter, 8p, and 18q for the *NotI*-*MseI* MS-AFLP analysis (4, 5). We soon obtained preliminary results indicating that the proposed detection method of DNA methylation alterations would allow the coverage of a lesser

number of genes than we expected. This was because *NotI* sites tended to cluster rather than spread evenly over the genome. For example, the chromosomal region of 18q contains 411 genes and 174 *NotI* sites. If one *NotI* site is present per gene, more than 40% of the genes will be examined for changes in DNA methylation. However, fewer than 80 genes were demonstrated to actually possess *NotI* sites in their promoter regions. This amounts to only 20% of the genes in that region. Assuming that the sensitivity of the *NotI*-*MseI* DNA microarray MS-AFLP method is 75% using oligonucleotide probes as we previously determined, we can analyze only 60 out of the 411 genes. Additionally, the methylation status of the promoter region of one gene may not necessarily characterize the methylation statuses of its neighboring genes or coincide reciprocally with the transcription of the gene. Therefore, we directly proceeded to Tasks 3 and 4 to identify the genes that exhibit differences in expression and copy number between the primary culture of normal mammary epithelial cells and established breast cancer cell lines.

We examined the expression of 127 genes in the chromosomal region of 18q21-qter in normal and cancerous breast cells and tissues. Rather than analyze the entire region of 18q, we analyzed the genes in the chromosomal region of 18q21-qter. By focusing our efforts on the genes located in cytobands where potential tumor suppressor genes are likely to be located (6), the total number of protein-coding genes for analysis was reduced from 223 to 140. We used two different techniques to examine gene expression: systematic multiplex RT-PCR (SM RT-PCR) and DNA microarray hybridization. We used the Illumina BeadChips for DNA microarray hybridization. We identified several interesting genes that exhibited differences in gene expression. Partial or entire loss of expression was observed in genes such as CCBE1, CCDC11, CD226, NP_115536.1, NP_689683.2, RNF152, SERPINB8, and TCF4 in a majority of breast cancer cell lines that were examined. An increase in gene expression was rare, but found with the transcription factor ONECUT2 gene in all the cancer cell lines. We further examined the expression of the selected genes from 18q21-qter by *real-time* qRT-PCR. We did this not only with the cDNA specimens from breast cancer cell lines that were previously used in the SM RT-PCR studies, but also with the cDNA specimens prepared from the matched pairs of normal and cancerous breast tissues from breast cancer patients. *Real-time* qRT-PCR experiments confirmed that the SM RT-PCR results obtained with the breast cancer cell lines were correct. Analysis of clinical specimens of breast cancer demonstrated that the gene expression of CCBE1, TCF4, NP_115536.1, and NP_689683.2 was down-regulated in the majority of clinical cases of breast cancer. We also performed copy number analysis by SM PCR and also by arrayCGH. We found homozygous deletions of the SMAD4 and ELAC1 genes in the MDA-MB468 breast cancer cell line.

In Year 2, we examined the expression of 273 genes located on the p-arm of chromosome 8 in breast cancer cell lines by SM RT-PCR and DNA microarray hybridization using the Illumina BeadChips. We observed frequent decreases in expression of approximately two-dozen genes and increases in expression of several genes on this chromosomal arm. These changes in gene expression of the cell lines were later confirmed by *real-time* qRT-PCR. Additionally and more importantly, we found that a number of these variations were also observed in the majority of clinical cases of breast cancer that we examined. These included down-regulation of the MYOM2, NP_859074, NP_001034551, NRG1, PHYIP (PHYHIP), Q7Z2R7, SFRP1, and SOX7 genes and up-regulation of the ESCO2, NP_115712 (GINS4), Q6P464, and TOPK (PBK) genes. We did not observe any genes that were homozygously deleted in the breast cancer cell lines examined by SM PCR and arrayCGH.

In Year 3, we examined the expression of 624 genes in the chromosomal region of 1p33-pter by DNA microarray hybridization using Illumina BeadChips. We also analyzed the

expression of some of the genes by SM RT-PCR. As opposed to the chromosomal regions of 18q21-qter and 8p, we did not find particularly interesting candidate genes that exhibited down-regulation in expression in the 1p33-pter region. Although there were genes that exhibited decreased expression in several cancer cell lines in comparison with primary cultures of normal epithelial cells, the frequency was low for most of the genes. The PLCH2 gene was down-regulated in a majority of breast cancer cell lines that were analyzed. However, the expression in breast tumor tissues increased. A few other genes, such as HES5 and AJAP1, seem to be down-regulated in a majority of cell lines and await further examination by *real-time* qRT-PCR for confirmation.

Rather than determining the methylation statuses of the promoter regions of the candidate genes with potential tumor suppressor activity, we proceeded to construct the cDNA expression constructs of many of these candidate genes in a eukaryotic expression vector, pcDNA3.1/V5-His. This decision was based on our belief that gene expression is more important than methylation of the promoter for gene functionality.

KEY RESEARCH ACCOMPLISHMENTS

We have just finished the third year of the 3-year project. The key research accomplishment for the entire period of research is the determination of changes in expression of the genes in the chromosomal regions of 18q21-qter, 8p, and 1p33-pter. We did the analysis not only by the DNA microarray hybridization method, but also by the SM RT-PCR method. This use of these two methods complemented each other. The DNA microarray hybridization allows quantitative measurement for moderately to highly expressed genes but often fails to detect weakly expressed genes. The SM RT-PCR method allows semi-quantitative detection of weakly expressed genes. Our study has identified more than a dozen genes that are down-regulated in gene expression in breast cancer cell lines and also in clinical specimens of breast cancer in the chromosomal regions. We have constructed eukaryotic expression constructs of these genes, and they are waiting to be tested for functionality in tumor suppression activity.

REPORTABLE OUTCOMES

We have already published the results obtained from the copy number and expression analysis of the genes in the chromosomal region of 18q21-qter (7). The PDF of the paper is included in the Appendices. We have submitted a manuscript describing the results from the copy number and expression analysis of the genes in the chromosomal arm of 8p and are waiting for the outcome of the peer review. The PDF of the manuscript is also included in the Appendices. We have not yet finished the SM RT-PCR analysis of many of the genes in the chromosomal region of 1p33-pter. Although the DNA microarray hybridization experiments of all the genes in the region did not identify any particularly intriguing candidate genes, SM RT-PCR may identify several genes that are down-regulated in breast cancer among the genes that are weakly expressed. Once the work is completed, we will publish the results. Additional funding will be necessary to perform DNA transfection experiments of the eukaryotic expression constructs of those candidate genes that have been identified in this study, and we are trying to obtain the funding to secure the continuation of this important research.

CONCLUSIONS

We have learned several important lessons from the completed research. First, methylation status of gene promoter does not always coincide with gene transcription activity. The genes with

hypomethylated promoter are occasionally unexpressed. On the other hand, the genes with hypermethylated promoter tend to be unexpressed as anticipated. Second, differences in gene expression between the primary cultures of normal breast epithelial cells and established breast cancer cell lines are often reproduced in the clinical specimens of normal and cancerous pairs of breast tissues. However, there are cases where the differences were reversed in the clinical specimens. The most prominent case was found with the PLCH2 gene, where the expression is down-regulated in all the breast cancer cell lines examined, but up-regulated in a majority of tumors. One possible explanation is that the changes in the cells other than epithelial cells in cancerous tissues may be responsible. Because this phenomenon may implicate an important interaction between breast cancer cells and normal cells surrounding them in tissue, we would like to determine the cause of this discrepancy and report it in future results.

The expression analysis of the genes in the chromosomal regions that are frequently deleted in breast cancer resulted in the identification of candidate genes with potential tumor suppressor activity. Both somewhat characterized genes and previously uncharacterized genes are found among them. Because the decrease or loss of gene expression, even if it is combined with the decreased copy number, is not sufficient to demonstrate the functionality in tumor suppression, we will need to proceed to examine the activity by DNA transfection of the expression constructs of those candidate genes.

BIBLIOGRAPHY

• Publications

Yamamoto, F. and Yamamoto, M. (2007). Scanning copy number and gene expression on the 18q21-qter chromosomal region by the systematic multiplex PCR and reverse transcription-PCR methods. *Electrophoresis*. 28: 1882-95.

Yamamoto, F. and Yamamoto, M. Identification of genes that exhibit changes in expression on the 8p chromosomal arm by the Systematic Multiplex RT-PCR (SM RT-PCR) and DNA microarray hybridization methods. Submitted for publication.

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REFERENCES

1. Hanahan, D., and Weinberg, R. A. (2000) The hallmarks of cancer. *Cell* **100**, 57-70
2. Courjal, F., and Theillet, C. (1997) Comparative genomic hybridization analysis of breast tumors with predetermined profiles of DNA amplification. *Cancer research* **57**, 4368-4377
3. Forozan, F., Mahlamaki, E. H., Monni, O., Chen, Y., Veldman, R., Jiang, Y., Gooden, G. C., Ethier, S. P., Kallioniemi, A., and Kallioniemi, O. P. (2000) Comparative genomic hybridization analysis of 38 breast cancer cell lines: a basis for interpreting complementary DNA microarray data. *Cancer research* **60**, 4519-4525
4. Yamamoto, F., Yamamoto, M., Soto, J. L., Kojima, E., Wang, E. N., Perucho, M., Sekiya, T., and Yamanaka, H. (2001) *Not1-MseI* methylation-sensitive amplified fragment length polymorphism for DNA methylation analysis of human cancers. *Electrophoresis* **22**, 1946-1956.

5. Yamamoto, F., and Yamamoto, M. (2004) A DNA microarray-based methylation-sensitive (MS)-AFLP hybridization method for genetic and epigenetic analyses. *Mol Genet Genomics* **271**, 678-686
6. Yokota, T., Matsumoto, S., Yoshimoto, M., Kasumi, F., Akiyama, F., Sakamoto, G., Nakamura, Y., and Emi, M. (1997) Mapping of a breast cancer tumor suppressor gene locus to a 4-cM interval on chromosome 18q21. *Japanese Journal of Cancer Research* **88**, 959-964
7. Yamamoto, F., and Yamamoto, M. (2007) Scanning copy number and gene expression on the 18q21-qter chromosomal region by the systematic multiplex PCR and reverse transcription-PCR methods. *Electrophoresis* **28**, 1882-1895

APPENDICES

1. Paper
2. Manuscript

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Received February 9, 2007
Revised March 19, 2007
Accepted March 20, 2007

Research Article

Scanning copy number and gene expression on the 18q21-qter chromosomal region by the systematic multiplex PCR and reverse transcription-PCR methods

We examined differences in copy number and expression of 127 genes located on the 18q21-qter chromosomal region of the breast and prostate cancer cell lines, using the systematic multiplex PCR and reverse transcription-PCR (SM PCR and SM RT-PCR) methods that we developed. Semi-quantitative data were obtained that were comparable in quality, but not in quantity, to data from DNA microarray hybridization analysis. In the chromosomal region where losses are frequent in breast, prostate, and other cancers, we detected a homozygous deletion of the SMAD4 gene in the MDA-MB-468 breast cancer cell line. We also observed partial or entire loss of expression in genes such as CCBE1, CCDC11, CD226, NP_115536.1, NP_689683.2, RNF152, SERPINB8, and TCF4 in certain breast and/or prostate cancer cell lines. An increase in gene expression was rare, but found with the transcription factor ONECUT2 gene in all of the cancer cell lines examined. Real-time qRT-PCR experiments confirmed these SM RT-PCR results. Further analysis of clinical specimens of breast cancer by real-time qRT-PCR demonstrated that the gene expression of CCBE1, TCF4, NP_115536.1, and NP_689683.2 was downregulated in the majority of clinical cases of breast cancer.

Keywords:

Chromosomal scanning, gene expression / Copy number changes / Systematic multiplex PCR / Systematic multiplex RT-PCR DOI 10.1002/elps.200700093

1 Introduction

During cancer progression, normal cells undergo many complex changes, both genetic and epigenetic, at either the nucleotide or (sub-)chromosomal level. Oncogenes and tumor suppressor genes play important roles in promoting and inhibiting carcinogenesis, respectively [1]. Proto-oncogenes are activated by gene amplification, up-regulation of transcription and activating mutations, whereas tumor suppressor genes are inactivated by loss of the genes, transcriptional silencing, and inactivating mutations. Therefore, the examination of copy number and expression may help to identify genes involved in carcinogenesis.

Hoping to eventually identify potential tumor suppressor genes of both breast and prostate cancers, we targeted the q21-qter region of chromosome 18 among the

chromosomal regions that have been reported to be frequently lost in cancer. In that region, a few genes (SMAD2, SMAD4 and BCL2) have been linked to carcinogenesis. SMAD4, homolog 4 of the *Drosophila* 'mothers against decapentaplegic' (Mad) gene, is a cancer predisposition gene with tumor suppressor activity. The germline mutations of the gene cause the familial juvenile polyposis, which is an autosomal dominant disease characterized by a predisposition to hamartomatous polyps and gastrointestinal cancer [2, 3]. In addition to the germline changes, homozygous deletion of the SMAD4 gene was prevalent in pancreatic carcinomas, and somatic mutations were identified in some of the carcinomas that lacked deletions [4]. Although SMAD4 inactivation was also found with breast, ovarian, and other cancers, it was distinctly uncommon (less than 10%) in other tumor types [5]. SMAD2 and BCL2 are not cancer predisposition genes; however, somatic changes have been revealed. BCL2 (B-cell leukemia 2) is a proto-oncogene and it was cloned from the junction of t(14;18) translocation characteristic of follicular lymphoma [6]. The BCL2 protein is localized in mitochondria and when overexpressed it interferes with programmed cell death independent of promoting cell division [7]. SMAD2, another homolog of the *Drosophila*'s Mad gene, may play a role as a tumor sup-

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Abbreviation: SM, systematic multiplex

pressor gene in a small fraction (less than 10%) of colorectal cancers. Therefore, we cautiously assume that additional cancer genes may be present in the region.

There are not many genes in the 18q21-qter region, approximately 140, so we used the systematic multiplex (SM) PCR and SM reverse transcription (RT)-PCR methods, which we developed for semiquantitative analyses of copy number and gene expression [8–11].

2 Materials and methods

2.1 Genomic DNA and cDNA

The following genomic DNA samples were used for the SM PCR experiments of the genes on the 18q21-qter region: a normal tissue and a primary tumor of breast from an individual with invasive ductal carcinoma and its metastasized tumor to lymph node, a normal and a primary tumor tissue of prostate from an individual with prostate adenocarcinoma, primary cultures of normal breast and prostate epithelial cells, and from six mammary (MCF7, MDA-MB-468, MDA-MB-231, BT-20, T-47D, and Hs-578T) and four prostate (PC3, DU145, LNCaP, and MDA PCa2b) carcinoma cell lines. The primary cultures (HMEC and PrEC) were purchased from Cambrex, and the cancer cell lines were originally obtained from ATCC. High-quality DNA preparations were confirmed by gel electrophoresis. Genomic DNA from MCF7, MDA-MB-468, and BT-20 breast cancer cell lines was also used in the arrayCGH experiments.

For the expression analysis by SM RT-PCR, we used the following RNA samples: a normal and a primary tumor tissue of breast from an individual with invasive ductal carcinoma, a normal and a primary carcinoma tissue of prostate, another normal prostate tissue, and a hyperplastic prostate tissue, five mammary (MCF7, MDA-MB-468, MDA-MB-231, BT-20, and T-47D) and three prostate (PC3, DU145, and LNCaP) cancer cell lines, and from primary cultures of normal mammary and prostate epithelial cells. Scarcity of degradation was confirmed with RNA specimens by gel electrophoresis. Total RNA was used to prepare cDNA by RT using oligo dT as a primer and Advantage RT-for-PCR Kit (BD Biosciences-Clontech). These RNA and cDNA samples were also used in the DNA microarray hybridization and real-time qRT-PCR experiments, respectively. Additionally, cDNA samples prepared from 12 matched pairs of normal and tumor breast tissues were used in the real-time qRT-PCR experiments.

2.2 SM PCR and SM RT-PCR experiments to measure and determine copy number and expression of the genes on the 18q21-qter region

The detailed experimental procedures to establish the SM (RT)-PCR system have been previously described [8–11]. Briefly, the genes were categorized into groups of approxi-

mately ten genes, and the concentrations of PCR primers in multiplex reactions were optimized to amplify different sizes of DNA fragments in single exons at similar band intensities using genomic DNA from normal human tissues as a control. Genomic DNA and cDNA from the human cells and tissues were used to examine the copy number and expression of the genes on the chromosomal region of 18q21-qter, respectively. After SM (RT)-PCR, small aliquots of reaction products were analyzed by an 8% polyacrylamide gel electrophoresis, followed by staining with ethidium bromide. The gel pictures were taken and saved in TIFF format, the band intensity was measured using the ImageQuant software (Amersham Biosciences) and normalized by adjusting the average band intensities of individual gels.

2.3 DNA microarray hybridization experiments to measure and determine copy number and expression of the genes on the 18q21-qter region

The copy number was also analyzed by DNA microarray hybridization using the arrayCGH method [12–14]. The changes in copy number were examined of three breast cancer cell lines (MCF7, MDA-MB-468, and BT-20) at Nimble-Gen (Madison, WI, USA). Genomic DNA from normal females was used as a reference for all the three hybridization experiments. Relative fluorescence intensity, which is indicative of relative copy number, was determined over the entire human genome with 385 000 isothermal long oligonucleotide probes tiled through genic and intergenic regions at a median probe spacing of 6000 base pairs.

The expression analysis was also performed by DNA microarray hybridization. Total RNA was used from a normal breast tissue, a normal prostate tissue, primary cultures of normal mammary and prostate epithelial cells, five mammary (MCF7, MDA-MB-468, MDA-MB-231, BT-20, and T-47D) and three prostate (PC3, DU145, and LNCaP) cancer cell lines. The same preparations of RNA that were used in SM RT-PCR were used in the microarray hybridization. Illumina's Sentrix Human-6 Expression BeadChips, which represented probes from the entire 23 000 RefSeq collection and an additional 23 000 other expressed sequences, were hybridized with the biotinylated cRNA that was prepared following the manufacturer's protocol. After hybridization and washing, the BeadChips were treated with streptavidin-Cy3, washed, dried, and scanned for fluorescence intensity, using a BeadStation 500 that was equipped at the DNA Microarray Facility at Burnham Institute for Medical Research. Raw data were generated and then normalized using the Beadscan 3.0 software. The unique 30X average redundancy feature of the BeadChips allows absolute signal detection of a single fluorescence. Other microarrays require the CGH-type hybridization using two different kinds of fluorescence followed by determination of relative signal intensity due to a wide variation in the amounts of probes printed on different slides.

Data for the genes and sequences on the chromosomal region of 18q21-qter were extracted from the results of the DNA microarray hybridization experiments and used for comparison with the results from the SM PCR and SM RT-PCR experiments.

2.4 Real-time qRT-PCR experiments to measure and determine expression of the selected genes on the 18q21-qter region

Real-time qRT-PCR was performed for several genes (CCBE1, CCDC11, CD226, NP_115536.1, NP_689683.2, RNF152, SERPINB8, TCF4, and DYM). The ubiquitously expressed DYM gene was used as a control. The subset of the cDNA samples that were used in the SM RT-PCR experiments and additional cDNA samples prepared from the 12 matched pairs of normal and tumor breast tissues were analyzed. The same primer pairs that were used in the SM RT-PCR experiments were also used in the real-time qRT-PCR experiments. Using the Power SYBR Green PCR Master Mix purchased from Applied Biosystems, the reactions were conducted using the Mx3000p system (Stratagene) under the default conditions, except that the annealing temperature was raised to 60°C instead of 55°C. Data were analyzed using the MxPro software installed with the equipment, and the Ct values were obtained for the individual reactions.

3 Results

3.1 SM PCR and SM RT-PCR analyses of the genes on the chromosomal region of 18q21-qter

Using control genomic DNA template, the optimal primer concentrations were determined to unify band intensity as previously described [8–11]. Out of 140 genes on the chromosomal region of 18q21-qter, 13 genes failed to amplify specifically and were excluded from the system. Together with 4 genes in the 18q12.3 region that neighbors the 18q21.1 region and 2 genes in 18q12.2, the SM RT-PCR system consisted of 133 genes in 12 sets (Sets A–L). The list of the genes is shown in Table 1. The nucleotide sequences and concentrations of the primers used in this study are also shown.

We examined the copy number of those genes in breast and prostate cells and tissues by the SM PCR method. Results are shown in the left column of Fig. 1. We found dozens of genes with decreases in band intensity in cancer cell lines, most evidently in MCF7 cells. Complete disappearance of band was observed with the SMAD4 gene (Set B) in the MDA-MB-468 cells, and a drastic decrease in band intensity was observed with ELAC1 (Set C) and PLEKHE1 (Set H) in MDA-MB-468 and RAX (Set F) in BT-20. We also observed an increase, which is suggestive of gene amplification, in the SLC14A1 gene (Set E) in MCF7 among others.

Band intensity was densitometrically measured for the SM PCR bands and the values were input into the table of genes aligned based on their chromosomal locations. The partial results are shown of a normal breast, primary culture of normal mammary epithelial cells, and three mammary carcinoma cell lines in gray scale (black and white for the lowest and highest band intensity, respectively) in the left column of Fig. 2.

We next examined the expression of those 133 genes in breast and prostate cells and tissues. Results are shown in the right column of Fig. 1. The PCR conditions were elaborated so that small amounts of genomic DNA would produce bands. The absence of at least one band indicated the least amount of contaminating genomic DNA in the cDNA specimens. We found that approximately 40% of the genes were ubiquitously expressed in a large amount. Similar band intensities of the ubiquitously expressed genes in the specimens suggested comparable quality and quantity of the cDNA preparations. We also observed that 17 were not transcribed in either normal or cancerous breast/prostate cells/tissues. The other genes were expressed in some, but not all, of the cDNA samples examined. Among them, several genes showed interesting expression profiles. For instance, the expression of CCDC11 (Set C) was completely repressed in three out of five breast and three out of three prostate cancer cell lines that were examined, whereas it was expressed in normal cells and tissues. On the other hand, the expression of the RNF152 gene (Set A) was repressed in prostate, but not in breast, cancer cell lines. Additional genes that exhibited down-regulation of gene expression include TCF4 (Set E), NP_689683.2 (Set F), SERPINB8 (Set G), CCBE1 (Set I), CD226 (Set K), and NP_115536.1 (Set K) genes in a majority of the cancer cell lines and PSTPIP2 (Set A) and KIAA0427 (Set H) in a minority of the cancer cell lines that were examined.

For quantification, band intensity was measured for the SM RT-PCR bands. Together with genomic DNA as a control, the results of a normal breast, a normal prostate, primary culture of normal mammary and prostate epithelial cells, and five mammary and three prostate cancer cell lines are schematically shown in a gray scale from black (weakest) to white (strongest) in the middle column of Fig. 2.

3.2 Comparison of the SM RT-PCR and SM PCR results with the results from DNA microarray hybridization

We compared the results from the SM RT-PCR experiments with the results from the DNA microarray hybridization experiments. We normalized data from the DNA microarray hybridization experiments using the BeadsCan 3.0 software. The average fluorescence signal intensities of beads for individual genes on the 18q21-qter region were extracted and gray-scaled, and are shown, side-by-side with the data from the SM RT-PCR experiments, in the right column of Fig. 2.

Table 1. Primers used in the study

Gene Name	Fragment size (bp)	Primer 1 sequence	Primer 2 sequence	Primer conc. (nM)
Set A				
ZNF532	175	TGAAGGGCCTCCAACTTGGGTAT	AGGACTGGCCACTTTCTTGTTTC	41
CDH7	160	CTGAGAAACCTCAACGTCATCCGA	CACCAGGATCAACATCGGCTTCTT	205
TNFRSF11A	146	ATGCCAGGATGCTCTCATTGGTCA	TGTGGATTTGCTTCCAGGCTCAGT	41
MBD1	133	TCCAACGAAGCAGGAAGCAGGT	CAACAGGGCTTCTGTGGAAGCTG	102
MBD2	121	CCAGGTAGCAATGATGAGACCCTTT	TGTTAAGCCAAACAGCAGGGTTCT	68
DCC	110	ACTACCCAACAACCACCTATGCTG	AGTGGGTGAGTTGGTCAACACAAG	102
RNF152	100	TGTCATCGCCATTCCACACACTTC	ACGCTCCTTGGAGATGGGCA	136
CXXC1	91	TGTTTGAGCAGGAGCGCAATGT	GGATCGTCTGGATCGTCTGGT	170
ONECUT2	83	GAACAAACGCCGTCAAAGGAGAT	TGAAGAAGTTGCTGACGTTGTGAG	102
MRO	76	CTGGTGTATGGAAGTGTATGACCCTGTGA	CCAGAACGACGGTCAGAGTCTTCA	102
PSTPIP2	70	TGAGGCTCAAGAATGTGAACGAATAAACT	TGACAGCTGATTCACATGTAACCACAATGC	102
Set B				
CAD20_HUMAN	175	TGAACAGCACTGTCCACAGCTA	AAGTCGAAGCTCTGTTCCGAGTCC	273
CDH19	160	AGGAGCCTATACAGGCAGTCTTTG	ATCCAGCTAATGACCCTGTTCCCT	68
SMAD7	146	CATCTTCATCAAGTCCGCCACACT	GCTGCATAAACTCGTGGTCATTGG	85
RKHD2	133	CAAGACGAAAGCACGACTGTGTGA	ACTGGACATGATGGCGTTCTCT	55
BCL2	121	AGCATGCGGCCTCTGTTTGATTTT	AGGCATGTTGACTTCACTTGTGGC	68
PMAIP1	110	GAATCTGATATCCAACTCTTCTGCTCAGG	TCAAATTGATGAAACGTGCACCTCCT	102
SMAD4	100	GCTGCTGCTGGAATTGGTGTGAT	TGATGCTCTGTCTTGGGTAATCCG	102
MAPK4	91	GACCACGACAACATCGTCAAAGTG	TGTACGCCACGCTGAACCTGAACA	102
SMAD2	83	AGCTTCACCAATCAAGTCCCATGA	AACAGTCCATAGGGACCACACACA	55
RAB27B	76	GCGAATGGAACAGTGTGTGGAGAAGA	CCCATCCAAGTTTCCAGAATTTCCACC	41
PIK3C3	70	CAGAGTCTGATTGATGAGAGTGTCCATGC	GGGCAAACCTGTGAATCTGTTCCACC	41
Set C				
ME2	133	GGAGAGAGAATTCTGGGTCTTGGAGA	TTCCACATCAATACACACTGGCAGG	100
ELAC1	121	ATGGACAAAGCAAAGGAGCATGGC	AGGCAACTGGTTTGTACCTCTGAC	100
NETO1	110	AGGAGCTACAGCTGACTTTGCAGATG	ACAGCTGTGATCCACAGTGTGGT	100
CCDC11	100	AGGAGCGGAAAGCACAGATTGCAT	GCTAATCGGTCTTCTCCAGAGTTT	125
CBLN2	83	GCTCATGGAAAGGGAAGACAAAGTGC	CCGAGAATGTGGAGTATTTCCAGCC	125
C18orf24	76	AGAAGCCTCCCAAAGAGCAAAGA	GAAGGAACACCATTGAACTCATCAAGT	250
Set D				
SERPIN2	151	TTCAACAAGGGACGGGCCAATTTT	GTCCAGTTCTCCCTGTCATAACACCT	83
ZNF407	138	CTGTACTCCCACACCGTGCT	GGAGCCCTCCTGGGTGTAGATGA	125
GALR1	126	ACAGACAGTTCTGGTGGTGGTTGT	GCGGTGATTCTGAAGAGGAAGGAA	67
WDR7	115	CACCTCAGCTGCGCTGCATTAAA	ACGTTGCGGTTGGAAGTCCAGAT	166
GRP	105	GAAGCTGCAAGGAATTTGCTGGGT	TGAATCCCACGAAGGCTGCTGATT	83
ALPK2	96	AGTTTAAAGCACTACACCAGTGAACAAGT	CAATGCTCGGCTGCTTCTGTTTCT	166
LIPG	88	ACTTGGGAGACCTCTTGAAGATCCAG	AGGTAGCTGCGAACTCCTTCCA	83
SLC39A6	75	GTTCTACTAAAGGCTGGCATGACCGT	AAGATACGCCAGCATGGCTGACAA	166
GALNT1	66	TCAGTGCTGGATAAAGCCACAGAAG	CTTCCATTGCACTCTCTAATGCTGGG	125
Set E				
XP_372695.2	192	GCATACCCAAGGACAAGGCCATTA	TGCGGGCTACACAAGATTGATTCC	58
POLI_HUMAN	176	TCTCCTTGTAACCGGGAACATCA	AAAGCAGACACAGCAGGGTTTGAA	115
Q96N33_HUMAN	161	TGGCCATGACCCACATGAGGATT	TGAAACAGAAGATGAACCTGATGACCAGGG	58
Q8TCD1_HUMAN	147	GGTGCAGACATGAATGGATTACCAACA	AGGTCAGAGACAATTACAAGGAAGATGC	87
Q7Z5E4_HUMAN	134	ATGCTCTGGCGTCTACTGCATTTC	GACACCCAATTCCTTCACTTGCCA	87
SLC14A1	122	CTTCTGTTTGGCCACGCTATTGTTCC	TCTTGGCTTGCAAGTAGAAGATGC	115
TCF4	111	ATGAGGACCTGACACCAAGAGCAGAA	GCTCTTTGAAAGCCTCGTTGATGTC	144
PIGN	101	TTTGGTGTTCCTCAATGGCCTGG	TGGTGCTTCAGCAACCTCACAT	115
FVT1	92	GGTGGGCAGGATCGTGTGTTGTG	CCCTTATGGCAAACCTGGATGCAG	115
NP_001008240.1	77	GTTAGCACCAGGCAAACACAGTCA	TCAACAACCTCTGCTGGTGAAGCC	115

Table 1. Continued

Gene Name	Fragment size (bp)	Primer 1 sequence	Primer 2 sequence	Primer conc. (nM)
SERPINB7	71	AGATTCTTGAGCTCAGATACAATGGTGGC	TTCAGAGAGGTCATTCTCAGGCAGC	87
KATNAL2	66	ACTGCCGACTTTCTGGATGTGCTA	GTATCTCTGAGCCAGATTCTTTGCGG	87
SEC11L3	62	GAGGCTTGTACAAAGAAGGCCAGA	TCTTGCTCTTCCCACCACGTCCTT	115
Set F				
XP_113971.3	193	AAATCCACATGCGGAAGCACACAG	CAGCTCTGGCGCTTGATGTGGC	188
ENSG00000188451	177	CACACCCTGACACAGCTTATTTCTGC	ATCTCCAGTAACCTTGGCCACCCTTC	63
TCEB3C	162	TCTGGCCACTAAGACGGAGCCGAAA	GCCGCTAAGTCTCTGGCAAAGT	38
NP_005594.1	135	CAGAAGCATCGCAAGCGGTTGAA	GGAGGAGATGAGGTCCGCGTAG	125
VPS4B	123	TAGGGACCACTCAGAACAGTCTCA	TCCTAACAGGCTGCATAAGGGCAT	63
STARD6	112	AAACCCAGCATATTCCAACTAGTGATGT	GAGGATGAAGTTTACTAAGTTGGAAGGCA	94
KIAA1468	93	TTGCTGCAAGCTTAGTGAGTGAAGA	ATACATTGGCCAACATGGCACCTG	125
NP_998767.1	85	TCCCTCAGCCACCAACATCCATTT	GAGAACCAGGACTGGCTGTGTC	94
TXNL1	78	GTTTCAGTCGAATCAAGGTGAAGAGGA	TGTTGCCTGGACTGGAGTACCAAT	125
RAX	72	GACAAGTTCCCGCTGGACGAGG	CTCCTTGGCTTTCAGACGCAGC	188
SERPINB10	67	ATCCAGAACTCTCCTGCCTGATGAC	ATAGGGCGTTCCACAGAATCATCC	94
NP_689683.2	63	AGTGGCTCGCCATGAGCAAGAAAT	CTCCAGTTGTGTCTCAATGTCCACT	94
Set G				
ZCCHC2	194	GAACACGAACGCTAATGGGACAGT	TGCCATTGCAAATGGATGGCAGAG	87
XP_371118.1	178	GATCAACGAGGAAAGCGACTACCA	CAAGGCTTCATTCTCTCGCTGGAA	87
C18orf12	163	TTCCTCCAACATGCATCGCTCAATC	CTCCCACTTTCAGCATTCTGGCTT	87
LOXHD1	149	TCTTTAACTGTGACTGCCTCATCCC	CTCATAGCCTGTTGTACGATGACTT	87
NP_066015.1	136	GTGGAGGAAGAGGCAAGCTGTTT	GGTTCTGCCTGCTCTGAACCAAGA	87
CPLX4	124	TGGCTGGAGATGATGTGGATTTACC	TCCAAGTCCATGTTCTGGAGATTCTG	87
HDHD2	113	GCACCTCTGATAGCAATCCACAAAGC	GTGGCTTTGGTATCTGTGGCATACTC	46
C18orf54	103	GCCAAGAGAAATCTAGAGCAGTGTACTGAA	CCCATGATCTGTCTGCTTCAAGTTTATCT	87
SIA8C_HUMAN	94	ATTTACCACCAAGTGGCAGGAGTC	CAGAGTCAGCTTGGTGAGCCCTT	173
ACAA2	86	CCCATGGCAATGACTGCAGAGAATC	TGCTGTGACTGCAGGGCATATT	58
SERPINB12	73	GGCACAGATCCTGGAATGAGGTA	TTTAGAGTGAGATGGCAGCAGCAC	58
SERPINB8	68	CAGAAGTTCTATCAGGCAGAGCTGGA	TGCTTCCTGCACTCTTCAGTGTCT	115
NEDD4L	64	TCGCCTTGACTTACCTCCATATGAAACC	CACGGCCATGAGAAGTTTCTCTCGTA	87
Set H				
PLEKHE1	195	AAGGAGAAGGAGAAACAGCAGCACCT	CACCTCTATCACATTGTGGCTCCT	115
SIA8E_HUMAN	179	TTCCACAAGCTGGAGAAGTGCC	TACTGCGGATGGAAGTAGTAGACAGC	144
MALT1	164	GAGGACAAGCAGGAAGTGAATGTTGG	TGCAATGAGTGATAATGCCCTGCTCC	46
C18orf26	150	TGGCTTGCTCTTACGCTTGCTGA	AGGGTGTTCCAGGTTTACAGT	46
KIAA0427	137	ACAGCTGCCTGAGATGATGACAGA	GCGTCAGAGGGTTCCAGCTGTTAG	58
MC4R	125	GACTCTGGGTGTCATCAGCTTGTT	TCACCAGCATATCAGCCACAGCCAA	46
MYO5B	114	TTCACCAGAGTGGAGCAGTTCAGA	AGGTACACAGGGAGCAGATAGCCT	87
ATP5A1	104	AAGTGGCTGTTATCTATGCGGGTG	CTGGCTGACGACATGAGACAAGAA	58
LMAN1	95	AACCGTCAGACTGGTCACTGGAAT	GCAGGTGCTCTTTGATGTCAATGAAGTG	87
SERPINB13	87	GATGGCTCTATTAGTAGCTCTACCAAGCTG	TTCTTTCTTAACTCCCTGTCCCATTGCC	115
FECH	80	ATCCAGTCAAACGAGCTGTGTCC	TTAGTCTCCCTGCAGACAGGATTGAC	115
IER3IP1	69	GTGGATTGGAGAAGAGCCGGAAT	TCACGGTTCTTACAGATCGAATAAGGTTCA	144
C18orf20	65	AGGTTACAACCAGAGACCTGAAGGA	TCCTCTTCTCTTTGACAACCATGTGGC	173
Set I				
ENSG00000182288	232	TGCTGCTCAAGCTCCATTACGA	GCCAGAGACAGGCATGAGAGATCAAA	50
Q8N7F0_HUMAN	214	TTTGAAAAGGAACATAAATGACCTGACAGA	TTTAGGTCTTTGAGAAATTGCCACAGTGTT	94
Q9H380_HUMAN	197	CCTGCATGGATTTGCATGTTTCCC	AGGTTAGAAGAATAAGAAAGGGAGTCTGGA	125
CCBE1	181	CCTGGTTCTTTGACTTCCTGCTA	TTCTTGGATGGTCATCTCCAGAGCC	63
NP_079490.1	152	GGAAATGAACTGGCTGGATGAAGATCTGA	TCATCTTCTTGCAAAGCCATTGGTATGT	188
NARS	139	AAGGTGGTGCCACACTTTCAA	TGCTCTGCCCGGTATGACTGAGCAAT	94
PIAS2	127	ACCCTTAACGACAAGCAGTACGTC	GTCAGGAATGTTACTTCCAGTGCTGG	94

Table 1. Continued

Gene Name	Fragment size (bp)	Primer 1 sequence	Primer 2 sequence	Primer conc. (nM)
SERPINB4	116	TGGAAGAGAGCTATGACCTCAAGGA	TTTAGATACTGAGAGACCGTGGCTCC	94
DYM	106	CAAATATGTGGAAGAGGAGCAGCCC	CTGGATGCTCTGTGGATTCCAGT	94
CCDC5	97	TCAAGACCTTCTCATGGAGAGTGTGAA	GGCCACCGCACTGTCAACCAAA	125
ENSG00000141690	89	TTGGACTTCCATCATCTCATCAACTACT	AACTGTTCCAGAGATTCAAGGTGG	94
C18orf24	82	ACCCGTAAAGAAGCCTCCCAAAGA	AGGAACACCATTGAACATCACAAGT	94
Set J				
NP_055728.1	235	ACTGGTGCCTGTGTATGTGAAGGT	AGCCAGGGTCATATTTCCCGTGTA	46
SALL3	200	ACAACGAGATCTCCGTCATCCAGA	ATCCTCGATAAACC CGCTGAATGG	87
ENSG00000196512	169	AACCCAGAATGCCTCTTCTCCTCT	AGCTTCCTTGCACTGGGTATAAG	58
SOCS6	155	TGGATCAGTCCGTGAATGGCTTGT	TGTGCCAGTGAGTCCACTGAAGTT	46
PQLC1	142	CAGTGGAGCAGCTTCTCGGACTACG	CCAGCATGGCTTCGGTCAGCACAG	115
CNDP2	130	GTTGAGCCAGACTTGACCAGGGAA	TTTCATTCTGGGAGTGGGCTCCGT	87
Q8N8S9_HUMAN	119	CTCTTGTTCCAGGCCCATCCAGC	AATCCGAAGGAGGTTCAAGGACTG	173
NP_997344.1	100	TAATCATTGGCTGCCTCCACTCCA	CTTGACGGCTGTCAACAAACAGGT	87
NP_872376.1	92	AAAGAGGGAGAGAAGGAACCAGGCT	AGGAACCTGGCCCTTCGGAAGTCT	87
NP_079057.1	85	CTGAATCAGATCCGTAAGCTCCAGAGG	GCAAGTGCCTGAGTTCAGTCTTAAGT	87
ENSG00000182671	79	ATTGCCAGAAAGAACCTGGCTTGC	AGCAAGCCTAATGAAGAGGCTCCA	58
ENSG00000176594	74	AATACTTCCTTGGTCTGTTGGGCCAT	TCAGAGCCAGCTGCTTAAGGAATGTG	58
CYB5	70	AAGCTGGAGGTGACGCTACTGAGAAC	TTGGACATTTCCCTGGCATCTGTAG	87
Set K				
NP_115536.1	185	GCCTTTCTTGAATTCTTTGTCTCCTGC	CGATCCATCAGAGTCCAGCAGATGTT	63
ZADH2	170	TTAAGCAGGAGTACCCTGAAGGTGTC	CAATGTTCTGCTTTACAGGCCGA	94
ZNF236	156	CGGCCGTTCCATTGCACGCTTGT	CCGCTTCATGTGCAGCTTCATGTT	50
CD226	143	GCCACATTGTTTCGGAACCTGGAA	TCTGCCATGGACCAAGTTGCAGTA	63
TXNL4A	131	ACAAGATTAAGTGGGCCATGGAGG	TCAGTAGCGGTACTTGGTGGAGTA	94
ATP9B	120	ATCTCCTTCACCGCACTGATCCTGA	GAGTGAGGACACGTAGCAGCCTAA	125
PARD6G	101	AATGACGAGGTCTGGAGGTGAAC	GTGACGATGAGGTTGTGGCTGTTG	312
GTSCR1	93	ACTCATCTACTGCAAGCTTGCCCC	ACTGACCATAGAGATGGTAGTGATGTCT	63
CTDP1	86	CAGATGTTTGGTGAAGAGCTGCCT	TCAGACATACTGGGCTGTCGCTTT	94
Q9NY04_HUMAN	80	CCAAACTGCCATTCCAGTCACTCA	CCTAGTAGAACAAAGAAAGCCCTGGAA	125
CNDP1	75	ATGATCCGGATGGATCCACCATT	AATTAGCACCACGCTCTTGTGGAC	94
FBXO15	71	ACCCTCTGACAGCTCTAGCTTCTT	GCACTCTTCTTCCGCATCAACGTA	94
Set L				
SDCCAG33	219	GCAACGATTGTGCCTCTCAGTTCA	TCCGGTTGCAGAGCTTACATTGGA	83
TXNDC10	171	GTGCTATGGAATCTACACAGCCGA	AATACATCCTTGGGCTCTGCACT	67
NP_079081.1	132	CAGCTCCCTCAAGAGTTACCTGTCA	TCTGTTCTGCCACCTCCTCTCTCT	125
Q96MY0_HUMAN	121	AAAGGTGCCATGCCAGAGAGATGA	AGGACAGAAGCAGTTTGCTGATGC	83
NP_997343.1	111	CAATCCTGGCGGTTACCTCAGCGG	CAGCGCGTCTGGAGTAGTTTCTTT	125
NP_054896.1	102	CGTACAGTATACGGAGAAGCTGCACA	CTCAAACCTGGGCTCAGTCTTCAAGCA	167
XP_058931.6	87	TGGTGGCTATTGATGTGGACATGG	TGCAGTCTTCATCTCCTGTGCAGT	83
RTTN	81	CCCAAACCTCAGAAGCAAACCCTCT	CAGGAAGAATTAAGGAGCTGCACGAG	167
MBP	72	AAGGCCAGAGACCAGGATTGGCTA	CCTTGAATCCCTTGTGAGCCGATT	208

We also compared the results from the SM PCR experiments with the results from DNA microarray hybridization experiments. We had not established the arrayCGH system in our laboratory, so we outsourced the arrayCGH hybridization experiments to NimbleGen, one of the pioneering providers of commercial services of the technique. We submitted the same genomic DNA from MCF7, MDA-MB-468, and BT-20 breast cancer cell lines that was used in our SM

PCR experiments. Data for the probes corresponding to the sequences on chromosome 18 were extracted, and $\log_2$ values of relative fluorescence intensity to normal female genomic DNA were plotted on the Y-axis along the chromosomal location on the X-axis (pter to qter from left to right). Results are shown in the upper panel (a) of Fig. 3. Similarly, $\log_2$ values were calculated of relative band intensity of those cell lines to normal female breast from the SM PCR data in

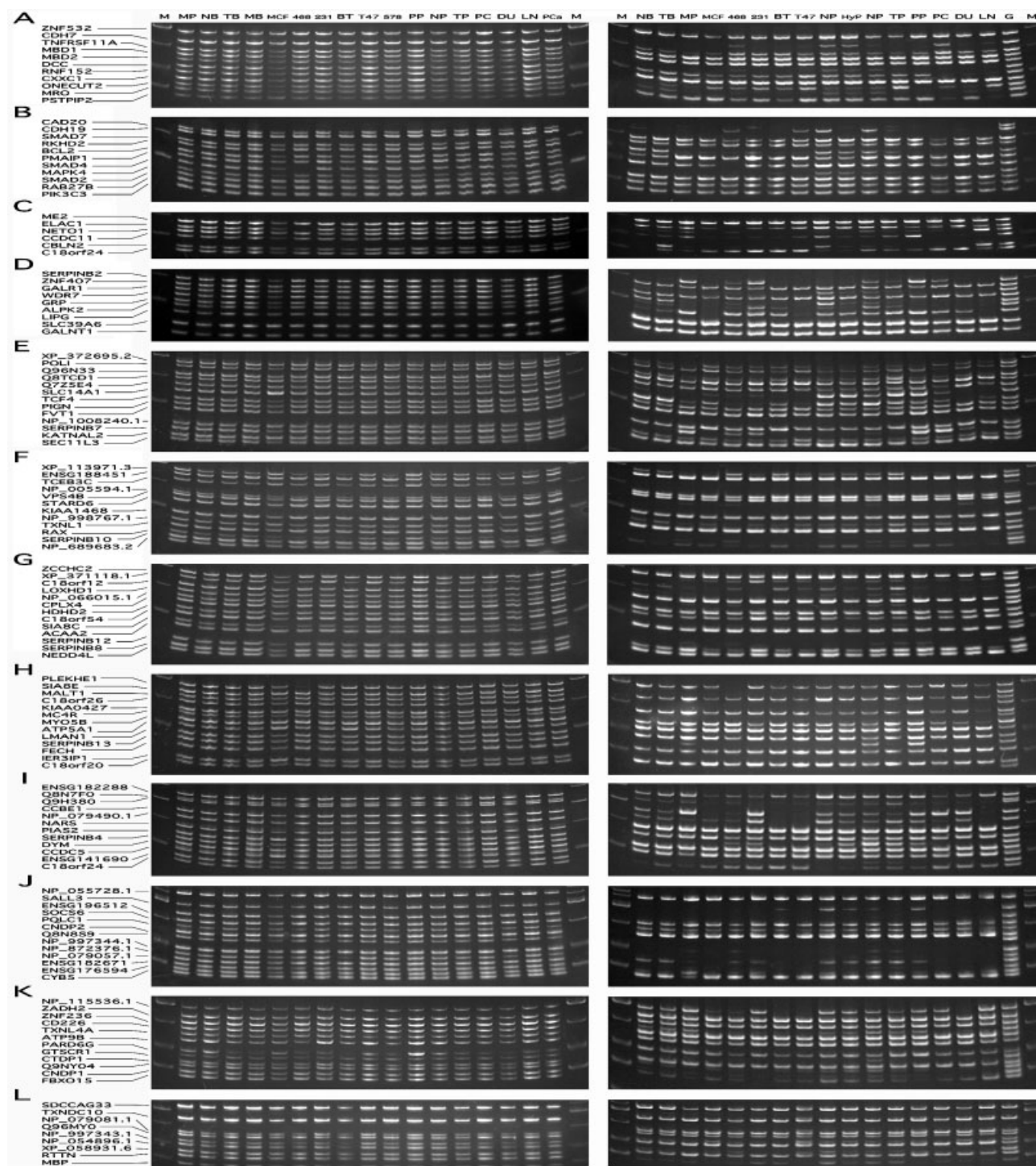


Figure 1. SM PCR and SM RT-PCR results of breast and prostate cells and tissues. The left and right panels show the results of the SM PCR and SM RT-PCR experiments, respectively. There are a total of 12 sets (A–L). SM PCR and SM RT-PCR were performed to examine copy number and expression changes in breast and prostate cancer cells and tissues. The sources of genomic DNA and cDNA are abbreviated: a normal sample (NB), primary tumor (TB), and metastasized tumor (MB) of breast tissue from an individual; a normal sample (NP), and primary tumor tissues (TP) of prostate from an individual; a normal prostate tissue (NP) from a third individual; a hyperplastic prostate tissue (HyP) from a fourth individual; primary cultures of normal mammary (MP) and prostate (PP) epithelial cells; and MCF7 (MCF), MDA-MB-468 (468), MDA-MB-231 (231), BT-20 (BT), T-47D (T47), Hs-578T (578), PC3 (PC), DU145 (DU), LNCaP (LN), and MDA PCa2b (PCa) cancer cell lines. The locations of the DNA fragments amplified from the individual genes are also shown at the left side of the gel pictures. The symbol M denotes DNA fragment size markers, and the symbol G shows the results of genomic DNA control in the SM RT-PCR experiments.

Gene Name	Band	Start Position	End Position	Str	SM PCR Results					SM RT-PCR Results										Fluorescence Microarray Hybridization Results															
					Str	NP	MP	MC	FC	Str	NP	MP	MC	FC	Str	NP	MP	MC	FC	Str	NP	MP	MC	FC	Str	NP	MP	MC	FC	Str	NP	MP	MC	FC	
GALT1	q12.2	31468599	31457920	D	133	192	190	168	149	294	528	543	500	458	672	586	496	527	573	413	498	448	158	169	119	77	343	135	90	155	284	325	186		
SLC39A5	q12.2	31943107	31963203	D	231	206	314	250	214	378	769	778	782	377	783	592	708	547	784	470	680	623	1046	1021	1957	105	231	108	363	1163	991	135	708	444	
PKC3	q12.3	37789187	37918442	B	348	351	182	268	198	217	227	287	280	170	181	344	367	332	278	388	164	310	287	279	874	176	290	483	190	285	418	391	388	270	380
SLC14A1	q12.3	41558157	41565046	E	255	221	478	239	192	278	338	18	49	22	43	33	33	15	572	49	43	19	21	21	21	21	21	21	21	21	21	21	21	21	
NP_590707.1	q12.3	41565046	41565046	E	172	188	192	105	94	240	104	38	53	23	49	38	104	70	92	90	40	23	22	22	22	22	22	22	22	22	22	22	22	22	
NP_059151.1	q12.3	41565046	41565046	E	181	190	152	146	145	321	425	551	553	333	628	443	498	380	501	407	477	359	448	330	230	344	359	75	139	115	219	137	181	101	
ATP11A	q12.3	41571701	41603221	A	103	101	94	98	288	250	251	555	450	159	275	309	408	216	355	229	288	237	237	237	237	237	237	237	237	237	237	237	237	237	
CCDC3	q12.3	41592868	41629279	I	208	221	342	288	260	317	392	210	339	382	417	438	484	380	287	332	478	489	151	151	422	239	289	176	283	130	487	385	484		
NP_059824.1	q12.3	42049765	42050844	E	230	236	229	182	248	283	208	442	589	318	497	537	307	235	480	380	424	292	151	151	422	239	289	176	283	130	487	385	484		
LOC610	q12.3	42151035	42150524	F	173	217	160	158	166	320	251	252	144	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150		
SIATSE_HUMAN	q12.3	42151035	42150524	F	173	217	160	158	166	320	251	252	144	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150		
PIAS2	q12.3	42151035	42150524	F	173	217	160	158	166	320	251	252	144	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150		
KATNAL3	q12.3	42151035	42150524	F	173	217	160	158	166	320	251	252	144	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150		
TCEB3C	q12.3	42151035	42150524	F	173	217	160	158	166	320	251	252	144	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150		
HOXD9	q12.3	42151035	42150524	F	173	217	160	158	166	320	251	252	144	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150		
ICR3P1	q12.3	42151035	42150524	F	173	217	160	158	166	320	251	252	144	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150		
OSPF1_HUMAN	q12.3	42151035	42150524	F	173	217	160	158	166	320	251	252	144	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150		
OTZP2_HUMAN	q12.3	42151035	42150524	F	173	217	160	158	166	320	251	252	144	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150		
ENSG0000018311	q12.3	42151035	42150524	F	173	217	160	158	166	320	251	252	144	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150		
ACAA2	q12.3	42151035	42150524	F	173	217	160	158	166	320	251	252	144	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150		
ACAA2	q12.3	42151035	42150524	F	173	217	160	158	166	320	251	252	144	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150		
ACAA2	q12.3	42151035	42150524	F	173	217	160	158	166	320	251	252	144	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150		
ACAA2	q12.3	42151035	42150524	F	173	217	160	158	166	320	251	252	144	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150		
ACAA2	q12.3	42151035	42150524	F	173	217	160	158	166	320	251	252	144	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150		
ACAA2	q12.3	42151035	42150524	F	173	217	160	158	166	320	251	252	144	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150		
ACAA2	q12.3	42151035	42150524	F	173	217	160	158	166	320	251	252	144	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150		
ACAA2	q12.3	42151035	42150524	F	173	217	160	158	166	320	251	252	144	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150		
ACAA2	q12.3	42151035	42150524	F	173	217	160	158	166	320	251	252	144	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150		
ACAA2	q12.3	42151035	42150524	F	173	217	160	158	166	320	251	252	144	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150		
ACAA2	q12.3	42151035	42150524	F	173	217	160	158	166	320	251	252	144	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150		
ACAA2	q12.3	42151035	42150524	F	173	217	160	158	166	320	251	252	144	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150		
ACAA2	q12.3	42151035	42150524	F	173	217	160	158	166	320	251	252	144	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150		
ACAA2	q12.3	42151035	42150524	F	173	217	160	158	166	320	251	252	144	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150		
ACAA2	q12.3	42151035	42150524	F	173	217	160	158	166	320	251	252	144	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150		
ACAA2	q12.3	42151035	42150524	F	173	217	160	158	166	320	251	252	144	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150		
ACAA2	q12.3	42151035	42150524	F	173	217	160	158	166	320	251	252	144	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150		
ACAA2	q12.3	42151035	42150524	F	173	217	160																												

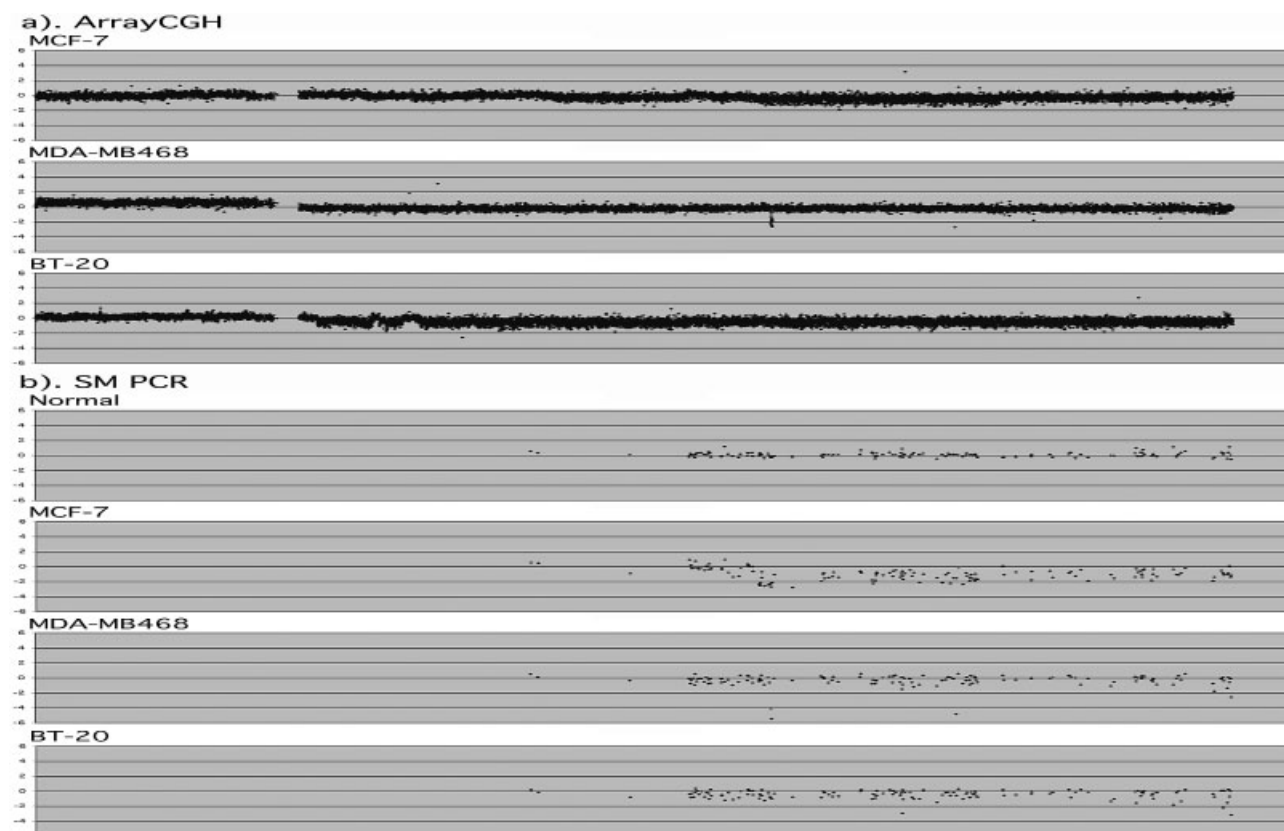


Figure 3. Comparison of the SM PCR results and the arrayCGH results. Data for the genes on chromosome 18 were extracted and normalized from the arrayCGH experiments of three breast cancer cell lines, MCF7, MDA-MB-468, and BT-20. The $\log_2$ values were calculated and plotted on the Y-axis with the chromosomal location on the X-axis in (a). The rightmost end of the X-axis corresponds to 80 000 000 base pairs from the pter. Since there are only 76 117 153 base pairs in the chromosome, there is a gap between the qter of the chromosome and the ends of the graphs. The relative band intensity was calculated by dividing the band intensity values shown in Fig. 2 of the genes for normal mammary primary cells, MCF7, MDA-MB-468, and BT-20 breast cancer cell lines by the corresponding values for a normal breast tissue. The $\log_2$ values were then used to plot on the graph in (b).

Fig. 2 and were plotted. Results are shown in the lower panel (b) of Fig. 3. Except for the number of data points, the graphs were similar between the SM (RT-)PCR and arrayCGH results. Both showed that the copy number was constant over the 18q21-qter chromosomal region in MDA-MB-468 and BT-20 cells, whereas there were at least two changes in copy number in the region in MCF7 cells: one around 46 Mb and the other around 60 Mb from pter. Additionally, homozygous deletion of SMAD4 gene was recognized by those two methods.

3.3 Comparison of the real-time qRT-PCR results with the results from the SM RT-PCR and DNA microarray hybridization

To confirm our findings by the moderately high-throughput SM RT-PCR and high-throughput DNA microarray hybridization expression analyses, we next performed real-time qRT-PCR for the promising candidates of cancer genes: CCBE1, CCDC11, CD226, NP_115536.1, NP_689683.2,

ONECUT2, RNF152, SERPINB8, and TCF4. As a control, we examined the expression of the DYM gene. This gene encodes Dymeclin (Dyggve-Melchior-Clausen syndrome protein) [15] and both the SM RT-PCR and the DNA microarray hybridization experiments showed ubiquitous expression in large quantity for all of the cells and tissues we examined. The $\log_2$ values of band/fluorescence intensity were calculated for SM RT-PCR and DNA microarray hybridization, using the data in Fig. 2 and plotted against the Ct values obtained by real-time qRT-PCR. The results were compared and are shown in Fig. 4.

Figure 4 clearly demonstrates a better correlation between the results of SM RT-PCR and real-time qRT-PCR than between the results of DNA microarray hybridization and real-time qRT-PCR. This is reasonable because both SM RT-PCR and real-time qRT-PCR are PCR-based techniques and the same pairs of primers that were proven useful in the SM RT-PCR were used in the real-time qRT-PCR experiments. Based on these results, we concluded that the differences in gene expression that we observed by SM RT-PCR were real.

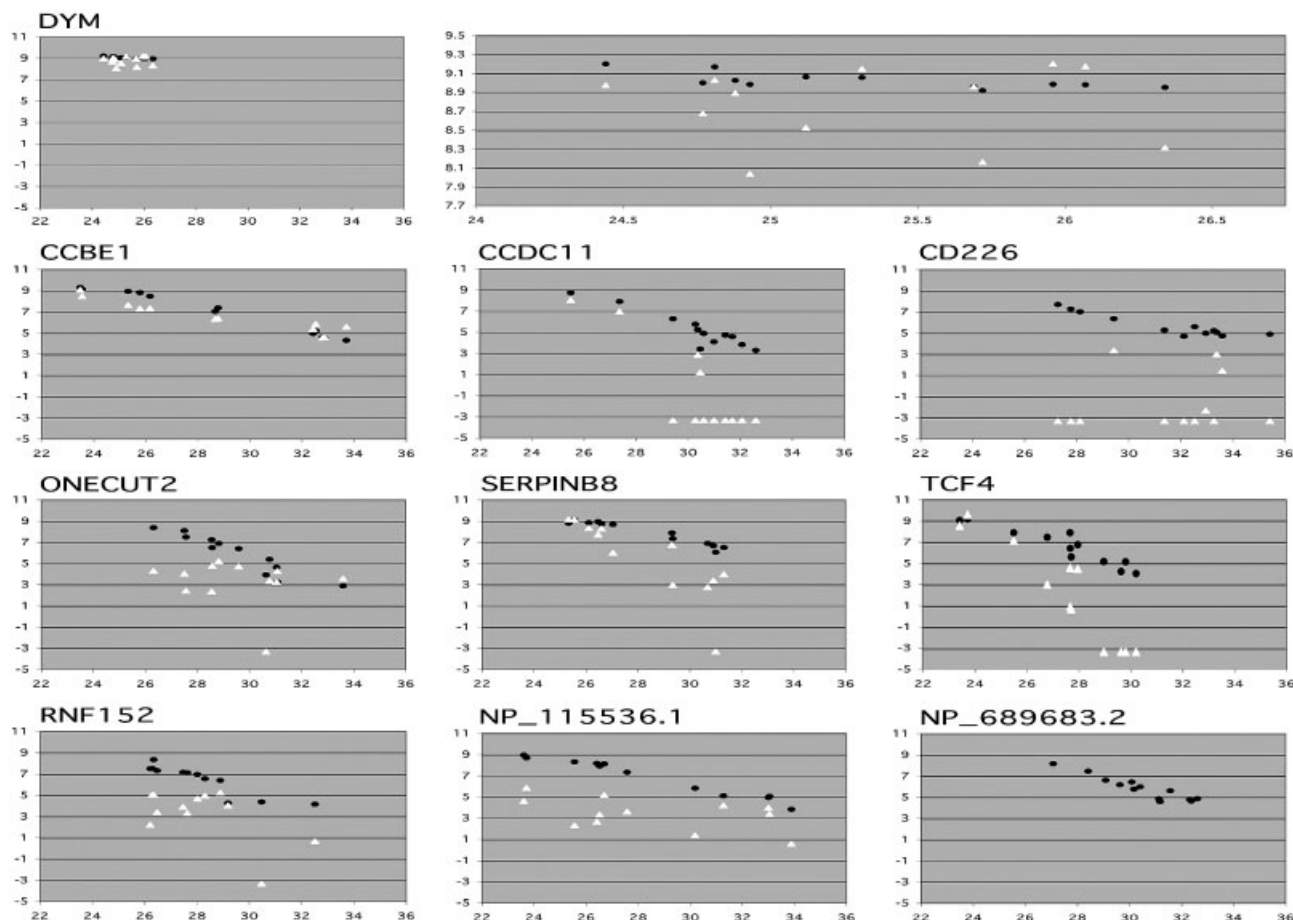


Figure 4. Correlation between the band intensity obtained from the SM RT-PCR or fluorescence intensity obtained from DNA microarray hybridization and the Ct values obtained from the real-time qRT-PCR experiments. The $\log_2$ values of the band intensity (closed circle) or fluorescence intensity (open triangle) were plotted along the Y-axis against the Ct values on the X-axis. The DYM gene was used as a control because this gene was ubiquitously expressed in large quantity in all the cells and tissues that were examined in both the SM RT-PCR and the DNA microarray hybridization experiments. Negative and zero values obtained by microarray hybridization experiments were assigned the value of 0.1 for these graphs. The portion of the DYM results was enlarged and is shown in the right graph on the top row.

3.4 Real-time qRT-PCR of clinical specimens of breast cancer

As the next step, we performed real-time qRT-PCR using cDNA prepared from clinical specimens of breast cancer. Cancer cell lines provide a useful starting point for the discovery and functional analysis of genes involved in cancer. The alterations found in cancer cell lines, however, may not necessarily be present in the original tumors. Those changes may have been acquired during a long cultivation *in vitro*. Therefore, it was necessary to evaluate whether the same differences are also observed in clinical specimens of cancer in addition to cancer cell lines. We did this using cDNA prepared from matched normal and tumor pairs of breast tissues. The expression of the DYM gene was used as a control to normalize the expression levels. The subtractive Ct values were plotted of the matched normal (on X-axis) and tumor

(on Y-axis) pairs of breast tissues and the partial results are shown in Fig. 5. Downregulation of gene expression was observed with the CCBE1, NP_115536.1, NP_689683.2, and TCF4 genes in a majority of clinical cases of breast cancer (11, 9, 9, and 11 out of 12 cases). A reduction of greater than 50% was observed in 8, 5, 7, and 9 cases, respectively.

4 Discussion

Using a model SM (RT-)PCR system that contained genes from autosomes and the X chromosome, we previously demonstrated that less than a twofold difference in copy number could be detected by SM PCR [11]. In the present study we applied SM PCR and SM RT-PCR to examine the changes in copy number and expression of more than a hundred of genes in breast and prostate tumors and cancer

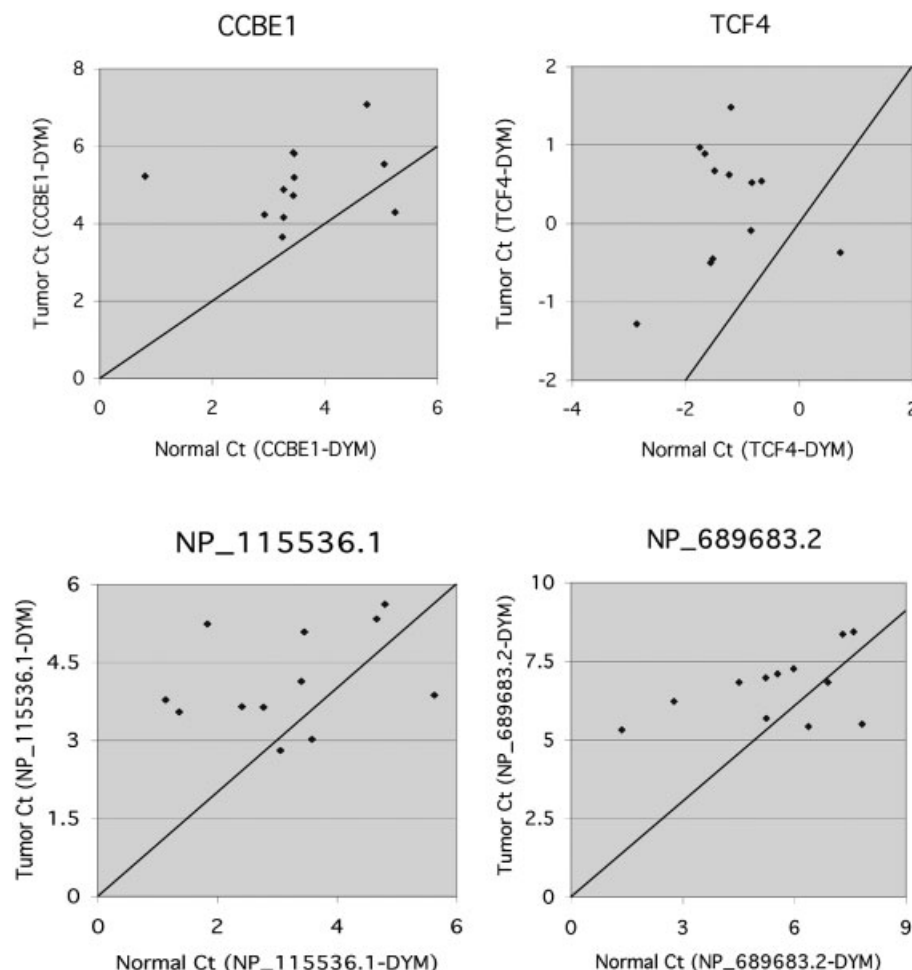


Figure 5. Expression of the selected genes in matched normal and cancer breast tissues. The gene expression was determined for the CCBE1, TCF4, NP_115536.1, and NP_689683.2 genes by real-time qRT-PCR using cDNA prepared from 12 matched normal and cancer breast tissues. The expression of the DYM gene was used to normalize the expression data. The subtractive Ct values (minus Ct DYM) of normal tissues are mapped on the X-axis and those of the corresponding tumor tissues from the same individuals are on the Y-axis. The line $y = x$ is also shown. The dots above the line indicate downregulation in tumor, whereas dots below indicate upregulation.

cell lines. The total number of genes analyzed by SM (RT)-PCR was 133 and exceeded the number of genes analyzed in any one of our previous studies. We focused on the genes on chromosomal region of 18q21-qter because loss of this region has been repeatedly observed in breast and prostate cancers, and tumor suppressor genes, whose inactivation could contribute to the development of breast and prostate cancers, have yet to be identified.

We observed a striking increase in band intensity with the SLC14A1 gene in MCF7. SLC14A1 is a member of SLC14 gene family of urea transporters [16]. Although the copy number increase of the SLC14A1 gene is associated with an increased gene expression, this difference is unique to MCF7, and therefore does not seem to be common phenomenon. Both the SM PCR and arrayCGH methods failed to detect the sequences of the SMAD4 and ELAC1 genes in the MDA-MB-468 cell line. This is in line with the expression studies since scarce or no expression is found in the SM RT-PCR and microarray experiments, suggesting homozygous deletion of the genes. Homozygous deletion was previously reported of the SMAD4 gene in pancreatic cancer [4] and of

the ELAC1 gene in a lung carcinoma cell line, Ma29 [17]. The results differed between the two methods with the RAX and PLEKHE1 genes. RAX is a paired-type homeobox gene, and the CpG island associated with the RAX gene promoter was found methylated in melanoma [18]. We observed considerably decreased signal only by the SM PCR method. However, the functional significance is not clear because the gene was rarely expressed in normal and cancerous breast and prostate cells and tissues. The PLEKHE1 gene, which is also called PHLPP, encodes a PH domain leucine-rich repeat protein phosphatase that specifically dephosphorylates the hydrophobic motif of Akt protein kinase, promotes apoptosis, and suppresses tumor growth [19]. An additional screening showed that MDA-MB175-VII, another breast cancer cell line, also failed to amplify the SM PCR band from the gene, in addition to MDA-MB-468. However, the following DNA sequencings determined that rather than a homozygous deletion of the PLEKHE1 gene, a 3-nucleotide deletion (GCA at nt 4743–4745) in those cell lines in one of the two primer sequences used in the amplification was responsible for the disappearance. The difference, which causes the

deletion of one of the two glutamines at amino acids 1580–1581, was not found in Ensembl or GenBank SNP databases and its functional significance remains to be elucidated.

The first difference we observed between the SM RT-PCR and DNA microarray hybridization experiments was that the results from the DNA microarray hybridization experiments exhibited a tendency to scatter to the lowest and highest extremes unlike the results from the SM RT-PCR experiments. This is reasonable because a wider linear range of signal detection by the hybridization method allowed the detection of stronger signals without saturation and also because PCR-based SM RT-PCR could detect signals from rare transcripts by amplification. The second difference was that there were eight genes whose expression was not detected by the hybridization method, but was detected by the SM RT-PCR method. For some of the genes it is possible that DNA fragments amplified by SM RT-PCR and the oligonucleotide probes in the BeadChips were derived from alternatively spliced different exons or that the expression was too low to be detected without amplification. However, we suspect that inadequate probes that were not pre-tested may have caused some of the false-negative results. In the SM RT-PCR experiments, we used normal genomic DNA template, which contains all the genes, to establish the system. The useless primers, which failed to amplify the expected sizes of DNA fragments or produced additional bands, were excluded. The problem with the DNA microarray hybridization method is that not all the probes in DNA microarray have been tested for their utility.

In contrast to copy number analysis, we observed more differences in gene expression. Decreased expression in tumors and cancer cell lines was observed with both known protein-coding genes, as well as uncharacterized potential genes. The known genes include coiled-coil domain-containing protein 11 (CCDC11), RING finger protein 152 (RNF152), T cell-specific transcription factor 4 (TCF4) [20, 21], cytoplasmic protease inhibitor 8 (SERPINB8) [22], collagen and calcium binding EGF domains 1 (CCBE1), CD226 antigen precursor [23], and PSTPIP2 that regulates F-actin bundling and enhances filopodia formation and motility in macrophages [24]. The uncharacterized genes include NP_689683.2, NP_115536.1, and KIAA0427. The down-regulation was confirmed for CCDC11, RNF152, TCF4, SERPINB8, CCBE1, PSTPIP2, and KIAA0427 by microarray hybridization, whereas the expression of CD226 and NP_115536.1 was undetectable in certain normal cells/tissues, in addition to several cancer cells, by the microarray hybridization method. The result of hybridization was not available for the NP_689683.2 gene. Consistently increased expression in cancer was only observed with the ONECUT2 (OC2) gene, which encodes a transcription factor characterized by the presence of a single “cut” domain and an atypical homeodomain [25], in the 18q21-qter region by SM RT-PCR. The increase was not so obvious by microarray hybridization, possibly because of its low level of transcription.

Real-time qRT-PCR confirmed that all the above-mentioned differences in gene expression of the cancer cell lines were real. However, only a subset of the genes survived candidacy after real-time qRT-PCR of clinical specimens of breast cancer. Downregulation in expression was observed for the CCBE1, TCF4, NP_115536.1, and NP_689683.2 genes in 11, 11, 9, and 9 out of 12 breast tumors, respectively. The results also showed that the CCBE1 and TCF4 gene were the most promising candidates among those examined, because decreased expression in tumor was observed at the highest frequency in breast cancer cases. As opposed to TCF4 whose link to breast cancer has recently been suggested [21], little is known about the CCBE1 gene and protein, except that the amino acid sequence of the CCBE1 protein predicts the presence of a signal peptide and collagen and calcium binding EGF domains. Because these domains are found in some of the extracellular matrix proteins, the loss of CCBE1 protein expression may result in changes in cellular characteristics, such as adhesion and motility. Further study is underway to pursue this possibility. The difference in gene expression was not so obvious with SERPINB8 (7/12 downregulated) and ONECUT2 (7/12 upregulated). Surprisingly, more tumors were found to exhibit increased expression than decreased expression with the CCDC11 (10/12 up), CD226 (9/12 up), and RNF152 (8/12 up) genes, in contrast to the decreased expression observed with the cancer cell lines. Heterogeneity in cellular constituency of tissues and contamination of normal cells and infiltrating lymphocytes in tumor tissues may have contributed to the results. The different environment that surrounds the cells *in vitro* and *in vivo* may have affected the gene expression. However, the discrepancy may also be explained by the acquisition of downregulation of the genes after the cancer cells were brought into the *in vitro* culture.

For the three previously identified cancer genes, we have mentioned SMAD4 above. Not much change was observed for SMAD2 in copy number and gene expression. For BCL2, the expression was found lower in both normal and cancerous cells in comparison with normal breast and prostate tissues. Whether cells other than epithelial cells are responsible for higher expression in those tissues or loss of 3-D architecture shut down transcription is a question that needs to be answered. In either case, it is unlikely that BCL2 plays an oncogenic role in breast or prostate cancer, as opposed to follicular lymphoma in which it does.

We started SM PCR and SM RT-PCR methods when the DNA microarray hybridization technique was still in its infancy. Only dozens of laboratories were successful in producing meaningful results. During the past several years, significant progress has been made. Commercial DNA microarrays with high quality have become available and the companies that perform custom hybridization have appeared. Concerns were raised about the reliability of DNA microarray results because the results varied con-

siderably among different platforms and different laboratories [26]. To improve the cross-platform concordance and to minimize the variation among laboratories, the Micro-Array Quality Control (MAQC) project was launched that recommended the use of standard RNA samples for comparison. Thanks to those efforts, DNA microarray techniques have been maturing. The results of the copy number analysis done at NimbleGen were satisfactory, although the turn-around time of 6 weeks was longer than we expected. The cost was reasonable. However, it was too expensive for us to order hybridization experiments with all 17 specimens analyzed by SM PCR, and therefore, we outsourced only the 3 hybridization experiments with the cell lines that were shown to exhibit significant changes in copy number by SM PCR. The number of detection points differed drastically between the two experiments. Whereas a little more than 5000 probes were examined over 18q21-qter with 6000 base pairs interval by the arrayCGH method using the NimbleGen microarrays, only 134 detection points were examined by the SM PCR method. In spite of this huge difference in number, both results exhibited impressively similar patterns of copy number changes. DNA microarray hybridization experiments for genome-wide gene expression were performed at the DNA Microarray Facility at the institute. The number of the probes of the Sentrix Human-6 Expression BeadChips was 47 293 and smaller than the number of the probes in the NimbleGen microarrays (385 000). Although each probe was represented by an average of 30 beads in the BeadChips, the confidence level may have been higher with the NimbleGen microarrays because the probes, which failed to hybridize with reference DNA, were excluded from consideration in the copy number analysis by arrayCGH. Compared with the hybridization using commercial DNA microarrays, establishment of the SM (RT)-PCR system is laborious and time-consuming. Because the results are obtained in multiple sets, there is a variation in the results among different sets. However, the situation may not be much different from DNA microarrays where the amounts of DNA printed may vary among different probes.

In the present study, we demonstrated the utility of the SM PCR and SM RT-PCR. We also verified that the results obtained by our methods were comparable in quality to the results obtained by DNA microarray hybridization method, although they were not identical. The ability to identify the amplified fragments by their size may counteract the deficiency of nonlinear amplification of signal by PCR and make the SM PCR and SM RT-PCR methods as useful as the DNA microarray hybridization method. It should be reiterated that we would have missed the opportunity of identifying both the CCBE1 and TCF4 genes as promising candidates if we had not performed SM RT-PCR. The results of DNA microarray hybridization using Illumina's BeadChips could be interpreted that the CCBE1 gene was expressed in all the cell lines and tissues examined, rather than that it not being expressed in some of the cancer cell

lines. This is because the values from 24 to 58 were non-zero and negative values were obtained with some other genes as shown in Fig. 2. On the other hand, the value of 2 for the TCF4 gene in primary culture of mammary epithelial cells could be interpreted as no expression and excluded. The results of SM RT-PCR showed high and near linear correlation with the results of real-time qRT-PCR, which demonstrated that the SM RT-PCR results do not require confirmation by real-time qRT-PCR. This is opposite to DNA microarray hybridization where the results always need to be confirmed.

We thank Dr. Kang Liu for technical assistance in DNA microarray hybridization experiments using the Illumina's Sentrix Human-6 Expression BeadChips and Mr. Lloyd Slivka for editorial assistance. We thank the Cooperative Human Tissue Network (CHTN) for providing normal and cancer breast tissues. This work was supported by the NIH grant 1R01CA087069 and the DOD BCRP grant W81XWH-05-1-0317 to FY.

5 References

- [1] Weinberg, R. A., *Ann. N. Y. Acad. Sci.* 1995, 758, 331–338.
- [2] Howe, J. R., Roth, S., Ringold, J. C., Summers, R. W. *et al.*, *Science* 1998, 280, 1086–1088.
- [3] Houlston, R., Bevan, S., Williams, A., Young, J. *et al.*, *Hum. Mol. Genet.* 1998, 7, 1907–1912.
- [4] Hahn, S. A., Schutte, M., Hoque, A. T., Moskaluk, C. A. *et al.*, *Science* 1996, 271, 350–353.
- [5] Schutte, M., Hruban, R. H., Hedrick, L., Cho, K. R. *et al.*, *Cancer Res.* 1996, 56, 2527–2530.
- [6] Tsujimoto, Y., Finger, L. R., Yunis, J., Nowell, P. C., Croce, C. M., *Science* 1984, 226, 1097–1099.
- [7] Hockenbery, D., Nunez, G., Millman, C., Schreiber, R. D., Korsmeyer, S. J., *Nature* 1990, 348, 334–336.
- [8] Yamamoto, M., Yamamoto, F., Luong, T.T., Williams, T. *et al.*, *Electrophoresis* 2003, 24, 2295–2307.
- [9] Yamamoto, M., Takai, D., Yamamoto, F., Yamamoto, F., *Gene Expr.* 2003, 11, 199–210.
- [10] Yamamoto, M., Yamamoto, A., Leung, P. C., Yamamoto, F., *Electrophoresis* 2004, 25, 2201–2211.
- [11] Yamamoto, M., Ahn, R.H., Yamamoto, F., *Electrophoresis* 2006, 27, 2529–2540.
- [12] Pinkel, D., Segraves, R., Sudar, D., Clark, S. *et al.*, *Nat. Genet.* 1998, 20, 207–211.
- [13] Pollack, J. R., Perou, C. M., Alizadeh, A. A., Eisen, M. B. *et al.*, *Nat. Genet.* 1999, 23, 41–46.
- [14] Albertson, D. G., Ylstra, B., Segraves, R., Collins, C. *et al.*, *Nat. Genet.* 2000, 25, 144–146.
- [15] El Ghouzzi, V., Dagoneau, N., Kinning, E., Thauvin-Robinet, C. *et al.*, *Hum. Mol. Genet.* 2003, 12, 357–364.
- [16] Shayakul, C., Hediger, M. A., *Pflügers Arch.* 2004, 447, 603–609.

- [17] Yanaihara, N., Kohno, T., Takakura, S., Takei, K. *et al.*, *Genomics* 2001, 72, 169–179.
- [18] Furuta, J., Nobeyama, Y., Umebayashi, Y., Otsuka, F. *et al.*, *Cancer Res.* 2006, 66, 6080–6086.
- [19] Gao, T., Furnari, F., Newton, A. C., *Mol. Cell* 2005, 18, 13–24.
- [20] Corneliussen, B., Thornell, A., Hallberg, B., Grundstrom, T., *J. Virol.* 1991, 65, 6084–6093.
- [21] Shulewitz, M., Soloviev, I., Wu, T., Koeppen, H. *et al.*, *Oncogene* 2006, 25, 4361–4369.
- [22] Gillard, A., Scarff, K., Loveland, K. L., Ricardo, S. D., Bird, P. I., *Am. J. Nephrol.* 2006, 26, 34–42.
- [23] Shibuya, K., Lanier, L. L., Phillips, J. H., Ochs, H. D. *et al.*, *Immunity* 1999, 11, 615–623.
- [24] Chitu, V., Pixley, F. J., Macaluso, F., Larson, D. R. *et al.*, *Mol. Biol. Cell* 2005, 16, 2947–2959.
- [25] Jacquemin, P., Lannoy, V. J., Rousseau, G. G., Lemaigre, F. P., *J. Biol. Chem.* 1999, 274, 2665–2671.
- [26] Tan, P. K., Downey, T. J., Spitznagel, E. L., Jr., Xu, P. *et al.*, *Nucleic Acids Res.* 2003, 31, 5676–5684.

Title:

Identification of genes on the p-arm of chromosome 8 that exhibit changes in gene expression by the systematic multiplex reverse transcription-PCR (SM RT-PCR) method

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Abbreviated title:

SM RT-PCR of genes on the p-arm of chromosome 8

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ABSTRACT

Losses of the p-arm of chromosome 8 are frequently observed in breast, prostate, and other types of cancers. Using the Systematic Multiplex RT-PCR (SM RT-PCR) method that we developed, we examined the expression of 238 genes located on the p-arm of chromosome 8 in five breast and three prostate human cancer cell lines. We observed frequent decreases in expression of two dozens of genes and increases in expression of several genes on this chromosomal arm. These changes in gene expression of the cell lines were later confirmed by *real-time* qRT-PCR. Additionally and more importantly, we found that some of the changes were also observed in the majority of breast cancer clinical cases that we examined. These included down-regulation of the MYOM2, NP_859074, NP_001034551, NRG1, PHYIP (PHYHIP), Q7Z2R7, SFRP1, and SOX7 genes and up-regulation of the ESCO2, NP_115712 (GINS4), Q6P464, and TOPK (PBK) genes.

Keywords:

Systematic Multiplex RT-PCR (SM RT-PCR), *real-time* qRT-PCR, DNA microarray hybridization, chromosome 8p, chromosomal scanning, gene expression, breast cancer, prostate cancer

1. INTRODUCTION

The activation of oncogenes and the inactivation of tumor suppressor genes both play important roles in carcinogenesis. Most changes in these activating/inactivating processes occur in copy number, gene expression, or nucleotide/amino acid sequences. Therefore, the determination of copy number and gene expression, together with nucleotide sequencing, assists in the identification of oncogenes and tumor suppressor genes. For the activation of an oncogene, a monoallelic dominant change is often sufficient. Examples of monoallelic activation include transcriptional activation of the BCL2 (B-cell leukemia 2) gene by the t(14;18) translocation that places this gene next to an active promoter in follicular lymphoma (1), MYCN gene amplification that is concomitant with an increased gene expression in neuroblastoma (2), and activating mutations in KRAS2 gene in cancers of lung, colon, pancreas, and others (3-5). However, for the inactivation of a tumor suppressor gene, haplo-insufficiency is rare and the disruption of both alleles (biallelic inactivation) is usually necessary.

Quantitative analysis of copy number progressed when the comparative genomic hybridization (CGH) method was invented based on the two-color fluorescence *in situ* hybridization (FISH) (6). In CGH, genomic DNA from a test sample is labeled with one fluorescent color, a reference genomic DNA is labeled with another color, and they are mixed and hybridized with metaphase chromosomal spreads of normal cells. The ratio of the two fluorescence intensities, rather than the absolute intensity, is used to monitor the difference in copy number. Using this technique, many maps of chromosomal alterations in cancer were produced. It was shown that there was a significant degree of heterogeneity among a variety of

tumors, as well as within the same type of tumor. Chromosomal gains and losses, which are indicative of the presence of oncogenes and tumor suppressor genes, respectively, were located on the chromosomes. For example, frequent gains in chromosomal arms 1q, 3q, 8q, 16p, 17q, 20q and losses in 1p, 6q, 8p, 13q, 16q, 17p, 18q, 22q, and X were reported in breast cancer (7, 8). Chromosomal losses were more frequent than gains in prostate cancer and observed with the chromosomal arms 1p, 5q, 6q, 8p, 10q, 13q, 16q, and 18q (9, 10). The use of BAC clone DNA microarrays (11, 12) and cDNA fragment microarrays (13, 14) for the CGH karyotyping analysis of copy number has produced a more powerful and high-resolution arrayCGH method.

Several tumor suppressor genes have been identified in the chromosomal regions of losses. These include CDH1 on 16q22 (15) and PTEN on 10q23 (16). The inactivation of those genes was considered to be the selective force that resulted in the loss of the corresponding chromosomal regions because of the frequent abnormalities and functional failure of the proteins encoded by those genes. Aiming to identify the novel genes with tumor suppressor activity, we started gene expression analysis. We chose the p-arm of chromosome 8 because this arm is one of the chromosomal arms most frequently lost in breast and prostate cancers, strongly suggesting that the region may harbor tumor suppressor genes involved in the pathogenesis of those cancers (8, 17). Although breast and prostate cancers both progress from an early, sex hormone-dependent, organ-confined disease to a highly invasive, hormone-independent, metastatic disease, they arise in two different organs. By pursuing tumor suppressor genes common to these cancers of two different organs, we speculated that the exclusion of inappropriate genes will be easier, whose expression is specific to either of mammary or prostate normal epithelial cells.

Here, we report the results obtained by analysis of gene expression on the chromosomal arm by the moderately high-throughput Systematic Multiplex RT-PCR (SM RT-PCR) method (18-21).

2. MATERIALS & METHODS

2.1 SM RT-PCR experiments to measure expression of the genes on the p-arm of chromosome 8

The following RNA samples were used for the gene expression analysis: a normal and a primary tumor tissue of breast from a patient with invasive ductal carcinoma, a normal and a primary carcinoma tissue of prostate from a patient with prostate cancer, another normal prostate tissue, and a hyperplastic prostate tissue, 5 mammary (BT-20, MCF7, MDA-MB-231, MDA-MB-468, and T-47D) and 3 prostate (DU145, LNCaP, and PC3) cancer cell lines, and primary cultures of normal mammary and prostate epithelial cells. cDNA was prepared by reverse-transcription of total RNA using oligo dT as a primer and the Advantage RT-for-PCR Kit (BD Biosciences-Clontech).

We followed the SM RT-PCR experimental protocols described previously (18-21). Briefly, the genes on 8p were categorized into groups of ~10 genes, and PCR primers were designed to amplify different sizes of DNA fragments from single exons of the genes in a group. After the multiplex reactions using genomic DNA from normal human tissues as a control, the concentrations of the primers were adjusted to produce bands of similar intensities. Once the conditions were elaborated, cDNA samples from the human cells and tissues were then used as templates to examine gene expression. Small aliquots of the SM RT-PCR reaction products were loaded onto an 8% polyacrylamide gel and electrophoresed. The gels were stained with ethidium bromide, the gel pictures were taken, and the images were saved in TIFF format. The band

intensity was measured using the ImageQuant software (Amersham Biosciences) and normalized by adjusting the average band intensities of individual gels.

2.2 DNA microarray hybridization experiments to determine gene expression

For comparison, the DNA microarray hybridization experiments were performed. Illumina's Sentrix Human-6 Expression BeadChips, which contained probes from the entire 23,000 RefSeq collection and an additional 23,000 other expressed sequences, were used. The following RNA samples were analyzed: a normal breast tissue, a normal prostate tissue, primary cultures of normal mammary and prostate epithelial cells, 5 mammary (BT-20, MCF7, MDA-MB-231, MDA-MB-468, and T-47D) and 3 prostate (DU145, LNCaP, and PC3) cancer cell lines. The same preparations of RNA that were used in SM RT-PCR were used in the microarray hybridization experiments. Following Illumina's protocol, biotinylated cRNA was prepared and hybridized with the BeadChips. After washing, the BeadChips were treated with Cy3-labelled streptavidin, washed, dried, and scanned for fluorescence intensity with Illumina's BeadStation 500. Raw data were generated and normalized using the Beadscan 3.0 software. The gene expression data for the genes on the p-arm of chromosome 8 were extracted.

2.3 *Real-time* qRT-PCR experiments to measure gene expression

Real-time qRT-PCR of the selected genes was performed using the same set of cDNA from the cells and tissues that were analyzed by the DNA microarray hybridization experiments, together with the genomic DNA control. The same preparations of cDNA that were used in the SM RT-

PCR were used in the *real-time* qRT-PCR experiments. In addition to this subset of the cDNA samples, additional cDNA samples prepared from 12 matched pairs of normal and tumor breast tissues were also analyzed by *real-time* qRT-PCR. The same primer pairs that were used in the SM RT-PCR experiments were also used in the *real-time* qRT-PCR experiments. The reagent from the Power SYBR Green PCR Master Mix (Applied Biosystems) was used and the yields of the PCR products were monitored using the Mx3000p system (Stratagene) under the default conditions, with the exception that the annealing temperature was raised to 60° C instead of 55° C. Data were analyzed using the MxPro software, and the Ct values were obtained for the individual reactions. The Ct values of the ubiquitously expressed ASAH1 gene, which is located on 8p, were subtracted from those values and normalized.

3. RESULTS

3.1 SM RT-PCR analyses of the genes on the 8p chromosomal arm

We established the SM RT-PCR system consisting of 254 genes. They were categorized into 26 groups. The list of the genes is shown in Table 1, together with the nucleotide sequences and concentrations of the primers used in this study and the sizes of the amplified DNA fragments. We examined the expression of those 254 genes in normal and cancerous breast and prostate cells and tissues. Results are shown in Figure 1. Because the PCR conditions were elaborated so that small amounts of genomic DNA would produce bands, the absence of at least one band was considered to confirm the absence of contaminating genomic DNA in the cDNA specimens. We found that approximately 42% of the genes were abundantly expressed in all of the cells and tissues that were examined. We also observed that approximately 30 genes were not expressed or rarely expressed in either normal or cancerous breast/prostate cells/tissues. The remaining genes were differentially expressed in some of the cDNA samples examined.

Among them, we identified a dozen genes that exhibited unidirectional changes in gene expression in both breast and prostate cancer cell lines. These include the GON1 (GNRH1) (set 16), NRG1 (set 18), PIWL2 (PIWIL2) (set 13), and Q7Z2R7 (sets 16 & 18) genes that were found down-regulated in all 5 breast and 3 prostate cancer cell lines and the ESCO2 (set 20), GSHR (GSR) (set 26), NP_115712 (GINS4) (set 23), Q6P464 (CDCA2) (set 16), TOPK (PBK) (set 17) genes that were found up-regulated in all of those cell lines, compared to the expression in normal epithelial cells. We also identified additional genes that exhibited changes in a

majority of either breast, prostate, or both cancer cell lines. Those include CH012 (C8orf12) (set 4), CHO13 (set 5), DEF1 (DEFA1) (set 1), EGR3 (set 20), ENST357748 (ENST000000357748) (set 11), FBX25 (FBX025) (set 1), MYOM2 (set 2), NP_065895 (set 7), NP_859074 (set 19), NP_001034551 (NP_1034551) (set 6), NPM2 (set 19), PHYIP (PHYHIP) (set 14), Q8NEP6 (set 5), Q96KT8 (set 6), SFRP1 (set 13), SOX7 (set 1), TPA (set 24), TR10D (TNFRSF10D) (set 15), and XR_017857 (C8orf48) (set 6) genes. We measured the intensity of the SM RT-PCR bands for quantification. The results were then aligned by the chromosomal locations of the genes and are shown in the left column of Figure 2. To facilitate the comparison, the intensity was shown in gray-scale from black (weakest) to white (strongest). Out of the 254 genes, 238 genes were mapped on the p-arm of chromosome 8 in the newest version of Ensembl (version 43) and the results of those 238 genes are shown.

3.2 DNA microarray hybridization analysis of gene expression of the genes on 8p

In order to compare the results from the SM RT-PCR experiments with the results obtained by an established method of DNA microarray hybridization, we performed the genome-wide gene expression analysis using Illumina's BeadChips. Data for the genes on 8p were extracted and aligned based on the chromosomal locations of the genes. Because the PCR primers for SM RT-PCR were designed based on the sequences that were not alternatively spliced, only the data using the "singular" or "all" probes that detect all the messages from the corresponding genes were extracted from the Illumina data. The average fluorescence signal intensity of >30 beads was extracted and gray-scaled, and are shown, side-by-side with the data from the SM RT-PCR experiments, in the right column of Figure 2. The expression data were obtained for the 230

genes on the p-arm of chromosome 8, 195 of which overlapped with the genes whose expression was determined by SM RT-PCR. Compared to SM RT-PCR, the results from the DNA microarray hybridization experiments exhibited a wider range of intensity as anticipated. There were 25 genes whose messages were not detected (fluorescence intensity below 10 in all the specimens). The number increased to 34 when the cut-off fluorescence intensity was set at 15.

3.3 Gene expression analysis of the selected genes by *real-time* qRT-PCR

We performed *real-time* qRT-PCR to re-examine the expression of the genes that exhibited consistent changes in expression by the SM RT-PCR method. The same set of cells and tissues that were analyzed by DNA microarray hybridization were examined for the expression of the CH012, CH013, DEF1, EGR3, ESCO2, FBX25, GON1, GSHR, MYOM2, NP_065895, NP_115712, NP_859074, NP_001034551, NPM2, NRG1, PHYIP, PIWL2, Q6P464, Q7Z2R7, Q8NEP6, Q96KT8, SFRP1, SOX7, TOPK, TPA, TR10D, and XR_017857 genes. Because of high expression of the messages in all the samples in both the SM RT-PCR and the DNA microarray hybridization experiments, we selected, as a control gene, the ASAH1 gene (22). This gene encodes N-acylsphingosine amidohydrolase, also called acid ceramidase (AC; EC 3.5.1.23), which catalyzes the synthesis and degradation of ceramide. The Ct values obtained by *real-time* qRT-PCR were plotted on the X-axis. The $\log_2$ values calculated of the measured band intensity and fluorescence intensity of the genes from the corresponding cDNA samples were plotted on the Y-axis. Results from all the selected important genes are shown in Figure 3. The results are shown on the same scale, and the result of the ASAH1 gene was also enlarged and shown next to the original figure on the top row. A higher degree of linearity was observed

between the results from the SM RT-PCR experiments and the results from the *real-time* qRT-PCR experiments than between the DNA microarray hybridization experiments and the *real-time* qRT-PCR experiments. This is reasonable considering that both the SM RT-PCR and *real-time* qRT-PCR are PCR-based and the same primers were used in those experiments. The differences observed by SM RT-PCR were confirmed to be real by *real-time* qRT-PCR, although some of them were not observed by DNA microarray hybridization.

We next examined whether the same differences in the expression level that were observed in the breast and prostate cancer cell lines were also present in the clinical specimens of cancer. Because we had matched normal and tumor pairs of breast tissues from a dozen breast cancer patients, we analyzed the expression of the selected genes in breast cancer by *real-time* qRT-PCR. To normalize the Ct values of the individual specimens, we subtracted the Ct values of the ubiquitously expressed *ASAHI* gene from those values. Down-regulation was observed with the following genes in a majority of 12 breast cancer cases: *MYOM2*, *PHYIP*, *SOX7* (10 cases), *DEF1*, *FBX25*, *NP_001034551* (9 cases), *CH012*, *GON1*, *NP_859074*, *NRG1*, *PIWL2*, *Q7Z2R7*, *SFRP1* (8 cases), *Q8NEP6*, *Q96KT8*, and *XR_017857* (7 cases). Similarly, up-regulation was observed in a majority of breast cancer cases with the following genes: *TOPK* (10 cases), *Q6P464* (9 cases), *ESCO2*, *NP_115712* (8 cases), *GSHR* (7 cases). For the remaining genes, down-regulation was observed in 4 (*NP_065895*), 5 (*TPA*), or 6 cases (*CH013*, *EGR3*, *NPM2*, *TR10D*). Many of the important results are shown in Figure 4, by plotting the subtractive Ct values of the tumor tissues on the Y-axis and those values of the normal adjacent tissues on the X-axis.

4. DISCUSSION

Previously we established the SM RT-PCR systems of families of glycosyltransferases (18), HOX homeoproteins (19), and integrins (20). We also gradually increased the size of coverage from a few dozen genes (21) to more than a hundred genes in a few cytobands (23). Here we attempted to establish the SM RT-PCR system of more than 200 genes on the entire arm of a chromosome. Excluding the DNA microarray hybridization approach, this SM RT-PCR analysis is one of the largest attempts to understand the expression of the genes on a chromosomal arm-wide scale. We aimed to incorporate as many genes as possible into the system. Because the defensin genes were highly homologous one another and possessed short coding sequences, we were unable to design primers for several members of the defensin gene family that selectively amplified single species of the gene members. Nonetheless, we included 254 genes in 26 multiplex reactions as shown in the primer list in Table 1 and the genomic DNA lanes (G) in Figure 1. However, the Ensembl database was not finished at the time that we retrieved the gene and sequence information. When we aligned our results in the most recent version, 43, we found that 11 genes that were previously mapped in the region did not exist any longer. Furthermore, a few dozen additional genes that were not previously mapped have been added. These include novel protein-coding genes, pseudogenes, miRNA genes, snRNA genes, and snoRNA genes. Because of this addition, Figure 2 has many open spaces, for which no expression data were available. 195 of the 238 genes whose expression was determined by SM RT-PCR overlapped with the genes whose expression was determined by the DNA microarray hybridization experiments. A generally good correlation was observed in the results between the SM RT-PCR and DNA microarray hybridization experiments, except that the expression of approximately 40

genes were detected only by SM RT-PCR. We think that the probes of those genes used in the DNA microarray hybridization were either inappropriate or not functioning as expected. The results shown in Figure 3 also illustrate this problem. To calculate the $\log_2$ values of fluorescence intensity from the DNA microarray hybridization results, we used 0.1 for the values below this number. Still, when the fluorescence signal was weak, as in the cases of NP_001034551 and PHYIP, no correlation was observed between *real-time* qRT-PCR/SM RT-PCR and DNA microarray hybridization. However, when fluorescence signal was strong, both SM RT-PCR and DNA microarray hybridization exhibited linear correlation with *real-time* qRT-PCR as shown with the GSHR, SFRP1, and TOPK genes.

In addition to the cell lines, we also examined the expression of the selected genes in the clinical specimens of breast cancer. As opposed to the *in vitro* cultured cancer cell lines that consist of a relatively uniform population of cells, tissues are made of several different types of cells and their ratios vary among different specimens. Therefore, measurement of the Ct values without standardization was not informative. We used the expression of highly and ubiquitously expressed ASAHI1 gene as a standard. By subtracting the Ct values of the ASAHI1 gene, we compared the relative ratios of the gene messages among different specimens. Rather than comparing the normal and cancer tissue specimens as two groups, we plotted the results from the individual pairs of cancer tissue specimens and their corresponding normal adjacent tissue specimens on the Y- and X-axes, respectively. The tendencies of up- and down-regulation in gene expression were easily confirmed with most of the genes examined. For several genes, the tendencies were not clear with the breast clinical specimens. Several potential reasons can be speculated. One possibility is that the cancer cell lines may have acquired down-regulation in

expression of those genes after they were brought into *in vitro* culture. Another possibility is that cells, other than cancer cells, that were present in the tumor tissues express these genes and losses/decreases in cancer cells may have been masked.

Among the genes that exhibited a matched tendency of up-regulation in the breast tumor tissues and breast cancer cell line cells, the tendency was striking with the ESCO2, TOPK, NP_115712, and Q6P464 genes. Because the ESCO2 gene is required for the establishment of sister chromatid cohesion during S phase of cell cycle (24), the TOPK gene encodes serine/threonine kinase that binds to the PDZ2 domain of Drosophila Discs-large (Dlg) tumor suppressor protein that regulates the cell cycle and/or cellular proliferation (25), the NP_115712 protein is a component of the GINS complex that is essential for the initiation of DNA replication (26), and the Q6P464 gene is associated with cell division cycle, up-regulation of these genes in tumors and cancer cell lines may simply be a reflection of a higher number of dividing cells in those specimens. These four genes were elevated in gene expression in all 5 breast and 3 prostate cancer cell lines that were examined, suggesting that this is a likely possibility.

Among the genes that were down-regulated in a majority of breast cancer cases, 4 genes, GON1, NRG1, PIWL2, and Q7Z2R7, were also down-regulated in all 5 breast and 3 prostate cancer cell lines that were examined. Additionally, 4 genes (NP_859074, NP_001034551, PHYIP, and SOX7) exhibited down-regulation in all the 5 breast cancer cell lines examined and 1 gene (SFRP1) exhibited down-regulation in a majority of breast and prostate cancer cell lines. Two genes, CH012 and XR_017857, exhibited down-regulation in a minority of cell lines, and 5

genes (DEF1, FBX25, MYOM2, Q8NEP6, and Q96KT8) showed decreased expression only in the breast cancer cell lines. Among these down-regulated genes, statistically significant tendencies were observed with the MYOM2, NP_859074, NP_001034551, NRG1, PHYIP, Q7Z2R7, SFRP1, and SOX7 genes, shown in Figure 4. The tumor-suppressing role has been well established of the NRG1 gene. The gene encodes neuregulin 1 (heregulin) that interacts with the NEU/ERBB2 receptor tyrosine kinase to increase its phosphorylation on tyrosine residues (27). The purified protein induces phenotypic differentiation of breast and prostate cancer cells and inhibits cell growth (28, 29). The SFRP1 and SOX7 genes play a similar role in carcinogenesis by repressing the Wnt signaling inside the cell. The SFRP1 gene encodes a secreted apoptosis-related protein that interferes with the Wnt-frizzled signaling pathway (30, 31). The potential role of the SFRP1 gene in tumor suppression of breasts and prostates was previously suggested (32, 33). The SOX7 gene encodes a transcription factor that possesses a functional transactivation domain in the C-terminus and significantly reduces Wnt/beta-catenin-stimulated transcription (34). The identification of three genes, NRG1, SFRP1, and SOX7, which are known to be involved in carcinogenesis among the candidates, indicates that the approach is working as expected.

In addition to those cancer-related genes, we also identified MYOM2, PHYIP, and three poorly characterized candidate genes. The MYOM2 and PHYIP genes encode myomesin 2, an M-band protein of sarcomeres (35), and phytanoyl-CoA hydroxylase-interacting protein (36), respectively. The NP_859074 gene predicts to encode a protein with the EF-hand domain (37), and the NP_001034551 and Q7Z2R7 genes predict proteins of 121 and 83 amino acid residues, respectively. Little else is known of those genes, however, the Q7Z2R7 gene is separated from

the NRG1 gene by only 1,577 bp and that the expression profiles of those genes were similar in the cells and tissues that were examined. Because the orientations of these genes are the same, there is a possibility that the Q7Z2R7 sequence may be transcribed run-off in the 3' untranslated region of the NRG1 gene messages rather than transcribed independently from its own promoter. Further studies will be needed before concluding that these candidates are genes with tumor suppressor activity. In summary, we have shown that the SM RT-PCR approach is successful in the identification of genes with altered expression through scanning of the genes at the subchromosomal level. It should be emphasized that by performing multiplex reactions of 10 genes on average, the number of reactions was reduced by 10 times in the SM RT-PCR experiments, as compared with *real-time* qRT-PCR of individual genes. Together with more flexibility in designing the SM RT-PCR system than DNA microarrays and using pre-confirmed primers, this advantage may allow the SM RT-PCR find its niche between the discovery method of high-throughput DNA microarray hybridization and quantitative *real-time* qRT-PCR.

ACKNOWLEDGMENTS

We are thankful to Dr. Kang Liu for technical assistance in DNA microarray hybridization experiments using the Illumina's Sentrix Human-6 Expression BeadChips and Mr. Lloyd Slivka for editorial assistance. We thank the Cooperative Human Tissue Network (CHTN) for providing matched normal and cancer breast tissues. This work was supported by the NIH grant 1R01CA087069 and the DOD BCRP grant W81XWH-05-1-0317 to FY.

REFERENCES

1. Tsujimoto, Y.; Finger, L. R.; Yunis, J.; Nowell, P. C.; Croce, C. M. Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. *Science* 226: 1097-9; 1984.
2. Kohl, N. E.; Kanda, N.; Schreck, R. R.; Bruns, G.; Latt, S. A.; Gilbert, F.; Alt, F. W. Transposition and amplification of oncogene-related sequences in human neuroblastomas. *Cell* 35: 359-67; 1983.
3. Capon, D. J.; Seeburg, P. H.; McGrath, J. P.; Hayflick, J. S.; Edman, U.; Levinson, A. D.; Goeddel, D. V. Activation of Ki-ras2 gene in human colon and lung carcinomas by two different point mutations. *Nature* 304: 507-13; 1983.
4. Shimizu, K.; Birnbaum, D.; Ruley, M. A.; Fasano, O.; Suard, Y.; Edlund, L.; Taparowsky, E.; Goldfarb, M.; Wigler, M. Structure of the Ki-ras gene of the human lung carcinoma cell line Calu- 1. *Nature* 304: 497-500; 1983.
5. Nakano, H.; Yamamoto, F.; Neville, C.; Evans, D.; Mizuno, T.; Perucho, M. Isolation of transforming sequences of two human lung carcinomas: structural and functional analysis of the activated c-K-ras oncogenes. *Proc. Natl. Acad. Sci. U.S.A.* 81: 71-75; 1984.
6. Kallioniemi, A.; Kallioniemi, O. P.; Sudar, D.; Rutovitz, D.; Gray, J. W.; Waldman, F.; Pinkel, D. Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. *Science* 258: 818-21; 1992.
7. Courjal, F.; Theillet, C. Comparative genomic hybridization analysis of breast tumors with predetermined profiles of DNA amplification. *Cancer Res.* 57: 4368-77; 1997.

8. Forozan, F.; Mahlamaki, E. H.; Monni, O.; Chen, Y.; Veldman, R.; Jiang, Y.; Gooden, G. C.; Ethier, S. P.; Kallioniemi, A.; Kallioniemi, O. P. Comparative genomic hybridization analysis of 38 breast cancer cell lines: a basis for interpreting complementary DNA microarray data. *Cancer Res.* 60: 4519-25; 2000.
9. Cher, M. L.; MacGrogan, D.; Bookstein, R.; Brown, J. A.; Jenkins, R. B.; Jensen, R. H. Comparative genomic hybridization, allelic imbalance, and fluorescence in situ hybridization on chromosome 8 in prostate cancer. *Genes, Chromosomes & Cancer* 11: 153-62; 1994.
10. Dong, J. T. Chromosomal deletions and tumor suppressor genes in prostate cancer. *Cancer & Metastasis Rev.* 20: 173-93; 2001.
11. Pinkel, D.; Seagraves, R.; Sudar, D.; Clark, S.; Poole, I.; Kowbel, D.; Collins, C.; Kuo, W. L.; Chen, C.; Zhai, Y.; Dairkee, S. H.; Ljung, B. M.; Gray, J. W.; Albertson, D. G. High resolution analysis of DNA copy number variation using comparative genomic hybridization to microarrays. *Nature Genet.* 20: 207-11; 1998.
12. Albertson, D. G.; Ylstra, B.; Seagraves, R.; Collins, C.; Dairkee, S. H.; Kowbel, D.; Kuo, W. L.; Gray, J. W.; Pinkel, D. Quantitative mapping of amplicon structure by array CGH identifies CYP24 as a candidate oncogene. *Nature Genet.* 25: 144-6; 2000.
13. Pollack, J. R.; Perou, C. M.; Alizadeh, A. A.; Eisen, M. B.; Pergamenschikov, A.; Williams, C. F.; Jeffrey, S. S.; Botstein, D.; Brown, P. O. Genome-wide analysis of DNA copy-number changes using cDNA microarrays. *Nat Genet.* 23: 41-6; 1999.
14. Pollack, J. R.; Sorlie, T.; Perou, C. M.; Rees, C. A.; Jeffrey, S. S.; Lonning, P. E.; Tibshirani, R.; Botstein, D.; Borresen-Dale, A. L.; Brown, P. O. Microarray analysis

- reveals a major direct role of DNA copy number alteration in the transcriptional program of human breast tumors. *Proc. Natl. Acad. Sci. U.S.A.* 99: 12963-8; 2002.
15. Oka, H.; Shiozaki, H.; Kobayashi, K.; Inoue, M.; Tahara, H.; Kobayashi, T.; Takatsuka, Y.; Matsuyoshi, N.; Hirano, S.; Takeichi, M. Expression of E-cadherin cell adhesion molecules in human breast cancer tissues and its relationship to metastasis. *Cancer Res.* 53: 1696-701; 1993.
 16. Li, J.; Yen, C.; Liaw, D.; Podsypanina, K.; Bose, S.; Wang, S. I.; Puc, J.; Miliaresis, C.; Rodgers, L.; McCombie, R.; Bigner, S. H.; Giovanella, B. C.; Ittmann, M.; Tycko, B.; Hibshoosh, H.; Wigler, M. H.; Parsons, R. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science* 275: 1943-7; 1997.
 17. Clark, J.; Edwards, S.; Feber, A.; Flohr, P.; John, M.; Giddings, I.; Crossland, S.; Stratton, M. R.; Wooster, R.; Campbell, C.; Cooper, C. S. Genome-wide screening for complete genetic loss in prostate cancer by comparative hybridization onto cDNA microarrays. *Oncogene* 22: 1247-52; 2003.
 18. Yamamoto, M.; Yamamoto, F.; Luong, T.T.; Williams, T.; Kominato, Y.; Yamamoto, F. Expression profiling of 68 glycosyltransferase genes in 27 different human tissues by the systematic multiplex reverse transcription-polymerase chain reaction method revealed clustering of sexually related tissues in hierarchical clustering algorithm analysis. *Electrophoresis* 24: 2295-307; 2003.
 19. Yamamoto, M.; Takai, D.; Yamamoto, F.; Yamamoto, F. Comprehensive expression profiling of highly homologous 39 Hox genes in 26 different human adult tissues by the Modified Systematic Multiplex RT-PCR method reveals tissue-specific expression

- pattern that suggests an important role of chromosomal structure in the regulation of Hox gene expression in adult tissues. *Gene Expr.* 11: 199-210; 2003.
20. Yamamoto, M.; Yamamoto, A.; Leung, P. C.; Yamamoto, F. Gene expression analysis of an integrin family of genes by Systematic Multiplex RT-PCR. *Electrophoresis* 25: 2201-11; 2004.
 21. Yamamoto, M.; Ahn, R.H.; Yamamoto, F. Scanning copy number and gene expression on the 16p13.3-13.2 chromosomal region by the systematic multiplex polymerase chain reaction and reverse transcription-polymerase chain reaction methods. *Electrophoresis* 27: 2529-40; 2006.
 22. Koch, J.; Gartner, S.; Li, C. M.; Quintern, L. E.; Bernardo, K.; Levran, O.; Schnabel, D.; Desnick, R. J.; Schuchman, E. H.; Sandhoff, K. Molecular cloning and characterization of a full-length complementary DNA encoding human acid ceramidase. Identification of the first molecular lesion causing Farber disease. *J. Biol. Chem.* 271: 33110-5; 1996.
 23. Yamamoto, F.; Yamamoto, M. Scanning copy number and gene expression on the 18q21-qter chromosomal region by the systematic multiplex PCR and reverse transcription-PCR methods. *Electrophoresis* 28: 1882-95; 2007.
 24. Hou, F.; Zou, H. Two human orthologues of Eco1/Ctf7 acetyltransferases are both required for proper sister-chromatid cohesion. *Mol. Biol. Cell* 16: 3908-18; 2005.
 25. Gaudet, S.; Branton, D.; Lue, R. A. Characterization of PDZ-binding kinase, a mitotic kinase. *Proc. Natl. Acad. Sci. U.S.A.* 97: 5167-72; 2000.

26. Takayama, Y.; Kamimura, Y.; Okawa, M.; Muramatsu, S.; Sugino, A.; Araki, H. GINS, a novel multiprotein complex required for chromosomal DNA replication in budding yeast. *Genes Dev.* 17: 1153-65; 2003.
27. Holmes, W. E.; Sliwkowski, M. X.; Akita, R. W.; Henzel, W. J.; Lee, J.; Park, J. W.; Yansura, D.; Abadi, N.; Raab, H.; Lewis, G. D.; et al. Identification of heregulin, a specific activator of p185erbB2. *Science* 256: 1205-10; 1992.
28. Peles, E.; Bacus, S. S.; Koski, R. A.; Lu, H. S.; Wen, D.; Ogden, S. G.; Levy, R. B.; Yarden, Y. Isolation of the neu/HER-2 stimulatory ligand: a 44 kd glycoprotein that induces differentiation of mammary tumor cells. *Cell* 69: 205-16; 1992.
29. Grasso, A. W.; Wen, D.; Miller, C. M.; Rhim, J. S.; Pretlow, T. G.; Kung, H. J. ErbB kinases and NDF signaling in human prostate cancer cells. *Oncogene* 15: 2705-16; 1997.
30. Finch, P. W.; He, X.; Kelley, M. J.; Uren, A.; Schaudies, R. P.; Popescu, N. C.; Rudikoff, S.; Aaronson, S. A.; Varmus, H. E.; Rubin, J. S. Purification and molecular cloning of a secreted, Frizzled-related antagonist of Wnt action. *Proc. Natl. Acad. Sci. U.S.A.* 94: 6770-5; 1997.
31. Melkonyan, H. S.; Chang, W. C.; Shapiro, J. P.; Mahadevappa, M.; Fitzpatrick, P. A.; Kiefer, M. C.; Tomei, L. D.; Umansky, S. R. SARPs: a family of secreted apoptosis-related proteins. *Proc. Natl. Acad. Sci. U.S.A.* 94: 13636-41; 1997.
32. Ugolini, F.; Charafe-Jauffret, E.; Bardou, V. J.; Geneix, J.; Adelaide, J.; Labat-Moleur, F.; Penault-Llorca, F.; Longy, M.; Jacquemier, J.; Birnbaum, D.; Pebusque, M. J. WNT pathway and mammary carcinogenesis: loss of expression of candidate tumor suppressor gene SFRP1 in most invasive carcinomas except of the medullary type. *Oncogene* 20: 5810-7; 2001.

33. Lodygin, D.; Epanchintsev, A.; Menssen, A.; Diebold, J.; Hermeking, H. Functional epigenomics identifies genes frequently silenced in prostate cancer. *Cancer Res.* 65: 4218-27; 2005.
34. Takash, W.; Canizares, J.; Bonneaud, N.; Poulat, F.; Mattei, M. G.; Jay, P.; Berta, P. SOX7 transcription factor: sequence, chromosomal localisation, expression, transactivation and interference with Wnt signalling. *Nucleic Acids Res.* 29: 4274-83; 2001.
35. van der Ven, P. F.; Speel, E. J.; Albrechts, J. C.; Ramaekers, F. C.; Hopman, A. H.; Furst, D. O. Assignment of the human gene for endosarcomeric cytoskeletal M-protein (MYOM2) to 8p23.3. *Genomics* 55: 253-5; 1999.
36. Lee, Z. H.; Kim, H.; Ahn, K. Y.; Seo, K. H.; Kim, J. K.; Bae, C. S.; Kim, K. K. Identification of a brain specific protein that associates with a refsum disease gene product, phytanoyl-CoA alpha-hydroxylase. *Brain Res. Mol. Brain Res.* 75: 237-47; 2000.
37. Katoh, M.; Katoh, M. Comparative genomics on FGF20 orthologs. *Oncol Rep.* 14: 287-90; 2005.

FIGURE LEGENDS

Figure 1. SM RT-PCR results of breast and prostate cells and tissues

The results of the SM RT-PCR experiments are shown. There are a total of 26 sets. SM RT-PCR was performed to examine gene expression changes in breast and prostate cancer cells and tissues. The sources of cDNA are abbreviated: a normal sample (NB) and primary tumor (TB) of breast tissue from an individual; a normal sample (NP), and primary tumor tissues (TP) of prostate from an individual; a normal prostate tissue (NP) from a third individual; a hyperplastic prostate tissue (HyP) from a fourth individual; primary cultures of normal mammary (MP) and prostate (PP) epithelial cells; and MCF-7 (MCF), MDA-MB-468 (468), MDA-MB-231 (231), BT-20 (BT), T-47D (T47), PC3 (PC), DU145 (DU), and LNCaP (LN) cancer cell line cells. The locations of the DNA fragments amplified from the individual genes are also shown at the left side of the gel pictures. The symbol M denotes DNA fragment size markers, and the symbol G shows the results of genomic DNA control.

Figure 2. Intensities of the bands amplified by SM RT-PCR and the intensities of fluorescence detected after DNA microarray hybridization of the genes on the p-arm of chromosome 8

Data that were obtained by the SM RT-PCR experiments that are shown in Figure 1 were used to prepare this table by the densitometry measurement of band intensity. In order to normalize the values, the average band intensities of individual gels were adjusted. The partial results of the

SM RT-PCR experiments are shown in the left column. The values of the band intensities of the PCR-amplified fragments were aligned by their chromosomal locations, and are shown in gray scale, with white as the strongest and black as the weakest. Data on fluorescence signal intensity were extracted for the genes on 8p from the DNA microarray hybridization results, normalized, and aligned. Results are shown in the right column. The gene names, cytobands, the starts and ends of the gene locations, and the primer sets, are also shown.

Figure 3. Correlation between the band intensity obtained from the SM RT-PCR or fluorescence intensity obtained from DNA microarray hybridization and the Ct values obtained from the *real-time* qRT-PCR experiments

The $\log_2$ values of the band intensity and fluorescence intensity were calculated and plotted along the Y-axis with black diamonds and gray squares, respectively, against the Ct values on the X-axis. The *ASAH1* gene was used as a control, because this gene was ubiquitously expressed in large quantity in all the cells and tissues that were examined in both the SM RT-PCR and the DNA microarray hybridization experiments. Negative and zero values obtained by microarray hybridization experiments were assigned the value of 0.1 for these graphs. The portion of the *ASAH1* results was enlarged and is also shown on the top row.

Figure 4. The expression of the selected genes in matched normal and cancer breast tissues

The gene expression was determined for the selected genes by *real-time* qRT-PCR using cDNA prepared from 12 matched normal and cancer breast tissues. The results from the genes that

showed consistent and meaningful changes in gene expression in both the cell lines and clinical specimens are shown. In order to normalize the expression data, we used the expression of the ASAHI1 gene as a control. The subtractive Ct values (minus Ct ASAHI1) of normal tissues are mapped on the X-axis, whereas those of the corresponding tumor tissues from the same individuals are on the Y-axis. The line $y=x$ is also shown. The dots above the line indicate down-regulation in tumor, whereas dots below indicate up-regulation.

Table 1. Primers used in the study

Set	Abbreviated Gene Name†	Frag. Size (bp)	Primer 1 Sequence	Primer 2 Sequence	Final Concn (nM)
1					
	ARHGA	144	TGAGAAGCAAAGCACGCCGGGC	TCTGCCAGACCATGACGGTCG	94
	SOX7	108	CATGGATCGCAATGAATTCGACCA	GGTGTACCTGGGAGACCGGAAC	94
	FBX25	95	CTCAGGACACCCCTGCACGGC	GCAGCCCTTAAACTTGAAGAGGTCGAT	141
	NP_078883	86	TCGACATCAGCTTGCCCGAGAA	CCAGTACGTCTGTCCATTGCACTCGTAG	469
	NP_079043	81	TGCCAGAATGGTACACAAAATCTTTTGG	TCTGGTTACTGAAGGAATCCCGGATCT	94
	CSMD1	77	CCAAAAGTTCAATACAATGGCTATGCTGG	TCATACATGGGGTTTTCAAACGATGC	188
	MTMR9	72	AAGCAAAAGTCAATATCCTTCGAAGGCAGT	AGGGACTCTCCTGCATCCCGTC	94
	DEF1	68	GGCCTGCTATTGCAGAATACCAGCG	CCTGGTAGATGCAGGTTCATAGCGAC	75
2					
	TNKS1	120	CAAATGCTCTTCTGTAGAGTGACCCCTGG	CAGCCCATTGACGCTCGGTCTAC	94
	NP_919260	109	CCCAAGGCCTACACCAACTCGG	AGTCGCAGGGCAGCGAGCTGT	469
	MYOM2	101	CCGAAGTGATTTGGTTCAAGAACGACC	GCCTTTGATGGTCATGCTGACGTACT	75
	PINX1	94	AGGCCCTGCTGGGACCAGAGTT	GGGCTTCAGGGTGAAGTCCCG	94
	DLGP2	88	TCCCTGGACCTGCCCGACAGAC	CGGAATTCTGCCGAAGGACG	141
	BLK	82	GAGCTGTACCGCGCGTCATC	GCACCGACTGCAGGAACTCGA	469
	D103A	78	ATTGCAGAGTCAGAGGCGGCCG	GCGTCGAGCACTTGCCGATCT	19
	MCPH1	74	TGTGAACTAGTCCACCTGTGCGGAG	TAGGGCCCGATGACGATGCTG	141
3					
	NP_940866	155	AGAACACCCAGGGATATACACCTCG	TGGCTCTGGGACTCCCGAGACT	54
	C8orf5†	119	TGTCTTGGGGTATCAGATTTACAGCGTAACA	CATTTTCCTGAAAATCTGCTGCAGATTAAAG	214
	Q96LV3	103	GGGGATTAAGTGGAGCTTATGGACTGC	TTTCAAACCCCAACCAAAATTCCTC	107
	CH014	94	GGTGGGAGGAGAGCCCGAGATC	CCCAGGTGAGACTTGCAAGTTCACG	161
	ENST297485	87	TGGCCATCTGCAAGATATGCCG	GGACATTTCTTGGAGCTGCTCGAG	64
	NP_1027009	82	GAAGCTGGAGCACAAATGTCCCG	TTCCACTTCCCAAGCTAAGCCTCG	43
	Q8NF75	77	CACAGGGTGTTATTTCCCATCTCATCG	AAGTCAGCCTCAGGATGCGGGT	536
4					
	Q8IWN7-2	170	GCACCTCCTCCTCCCGAAGAGT	CAACTGCTTCACAGGAAAGCGCA	75

XKR5	157	CCCGACACCATGGCCGACATT	CACCAACAGCTGCATGGTGACTTAGC	75
NP_778250	146	GGATGACGCCAAGGGCTCGAC	ACTCCGCCTCCTCCGGGCTAT	75
C8orf15+	135	AGCAGGTAGCACTGGAGCCGATC	GCAGTTCAGTGTCCCGTGGTC	75
CH012	82	CAGAAGTACAAGGTGAAGAATGCATACCGA	CCAAAGAAGCAAAAGTGCTAGCACCA	125
NP_689484	78	GCCCCTAATGGACCTGCATGGT	CAAAACCGACAGCTGGTATCGTGG	125
5				
NP_1035121	172	GGAGCTGCTGCAGCGCCAGAT	CTTCGCTTTCTTTCTTTTGCTCGC	107
Q86YV5	152	ACCCCATCAAGCGTATCCGCA	CAGGGCCCGCTTCATGTTCGAT	536
CH013	132	TGCTCAACGATGCCACCTACGA	TGATGTTTCATCTTGGTCACGCCAATA	161
XKR6	120	TGACATGCCAAGAAAGCGATACCC	TCTCGATATCGAATGCCTACTGCGG	107
Q8NEP6+	101	CAAATATGGATTTGGGGTGTGCGTAA	TGTCCTGCAGCCGCACCGATA	214
Q8N852	91	TGGGCTTCCTGGGACTCGGTG	GCTTGGAACCACAAAGAATGACGC	64
Q8TCU9+	85	GAAGCCAGGGGAACAAGGTTAAAAGG	TGTCACCATCTTCAGCCAAGCCAC	107
6				
MSRE	158	GAGCAACATGGAGAAGAGAATCCAGC	TCCCATGTCCCTGGACTGAGGAAA	102
XR_017857	145	GCCCTGTCTGCCTTTCTGAAACAA	GGTCATCTGAAAGCCTGGGAAAGT	102
649548	124	AGCCACAAGTGCTGCTGATGTGTA	GGATTGTTTACCCTGATGGCCAGA	68
FDFT	112	CCGGAGAATATTGACTTGGCCGT	ACACACTCTGGTTTCTGAGTCTCG	68
730602	103	GAATATTGGATGGATCCTGAAGGCGA	CAATGACAGAAATCTGTTCTTCAGCTGGC	68
Q96LV6	90	TGTTTGTGCCTAGCACGATTGGG	AGATGGTCCTGGGTCTCTGAATAC	102
NEIL2	84	AGAAGTTCCATCGAGGACAAGCCT	AGAAGTATCTCTGGTCCAGCAGTGT	102
Q96KT8	78	AAGCTTGTCTTTGCCTTCACGCC	CCCGGACCTCTTCTGATAAGGAAT	205
SGCZ	74	ACAGTGTATGAACTCTGCGTCTGC	TGACAAGTGGAACTACTCTGCT	102
NP_1034551	71	GCCTGTGCTTCCTTCAGAGACTCA	GGAGTGAAATGAAATAGTGCGGTCC	205
TUSC3	68	TCAGACCACCCAACTACTCTGGTA	AAGCAAACCTCCAACAAGCGACAC	102
7				
NP_1027009	138	GCCACTTTTACTGTGTCTCCCAT	AAATGCCACTGCCACTGCTATCTG	136
GATA4	125	TCTCAGAAGGCAGAGAGTGTGTCA	CCGGTTGATGCCGTTTCATCTTGT	102
BLK	114	GGATGGTCTATGCCAGAGGCTGA	AGTTTCCTGACCAGCCTGAGAGA	102
CH013	105	CACGTGGAAGAAGTGGGTGCAGAAG	GGTCTCCAGCCTCAGTTTCTCGGT	55
Q96LV6	97	TGTGGCTGTTGGACGCCTGTC	CAGAGAAAGACGCAGAGTGGAAGT	205
NP_065895	90	TCCACAGCCTTTATGCGCTACTAC	CAGGATACGGAGCTCTGACACATTC	136
CATB	78	AACACGTCACCGAGAGATGATGG	AGTAGGGTGTGCCATTCTCCAC	68
NEIL2	74	CAGAAGGGCCGTTGGTGAGGAAAT	CCCTGTCTTGACCACCTGCTGA	68
CH014	71	CCCTGAAAGTAGCAGGACAGCCTTA	CCTGGACTCACAGACTAGACTCTTGC	68

BLK	68	ACAAGCATTTCGTGGTGGCTCTGT	CTTCAGCATCTGCAGGTCCCATCATT	68
CH012	65	AGTGGAGGCCGGTAGTCTGAAT	TGAGAGAGGCAGGCGGAAGTCTTT	68
8				
Q86YV5	100	GGAAGAGGACCATCGGACGATCTA	TTCACCTACCTCGGAGTTCTGGCT	188
NP_004216	91	CAGTGTCAGATCAACAGCCATGT	ACTCACAACCACAGGAACCTTCCCTC	75
THEX1	83	GCCTCACTGTGGTCTTGATGACTCTA	TGATTCTGGAGTTCACACCCATCCT	125
NP_078883	70	TGCGCCAAGAGAGGTTTGCCTTTA	AGCTGAATACAAGGCCTCAGTGGT	75
MSRA	65	ACATCCGGGAGGGACAGACTTTCTA	GTTCTTGCTCAGGTACTGCTGGT	75
NP_919260	61	TGATGACGCTGGGCATGGTGTT	AGGGTGCCGGTCAGGTTGA	313
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PNMA2	178	TCATGCACATAGTGCAGGCAGACA	AGCAGGGTTTCTAGCCGTAACAC	167
LOXL2	163	ACTCCTGATATGCCGTACTTGGACC	GGGAAGTCTGGGTCTCGTTTGTTT	67
NP_060561	149	TGGTGGCTGGGAAACATTCTTGTC	TCCCATAACATGCAGCTCTCGTA	83
ADEC1	136	CCCAAAGGATTTTCACTACATCTTGCCG	TTCTAGAAGGTGGTTCACACACAC	83
WRN	124	AATTGGCATGCACTTATCCCAAGCGG	TGAGTTGACGGGAGGGTTTCGGAT	167
FGL1	113	GTGTCACTCTGCAAACCTGAATGG	CAGATTTAGAGAATACCACCACCCA	125
VATB2	103	ACATTGGCTGGCAGCTACTCCGAAT	TAATGCTTTGCAGAGTCTCGAGGG	83
D104A	86	TATGTGGTTATGGGACTGCCCGTT	GCATGCATAGGTGTTGGGACATCTTC	83
IKKB	73	CATGAATGCCTCTCGACTTAGCCA	AGGTAAGCTGTTGGAGGCCGT	167
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TEX15	195	GACCACAGTTGCAAGTACTGCCC	GCTTGTGGTAATGGCTGATGAGAAGC	41
MCPH1	179	GTTGTAGACAGGCTGGGAAAGAAGAC	GGAAGTGGTACCTTTGTTCTGTGTGC	68
2ABA	164	AGAAACACAAAGCGAGACATAACCCT	TGCCAGGCTGTGTGAAGGATTT	136
ADAM2	137	CATTTACCATTCCAAACCAATGAGATGGC	CGCTTGAATAGTCCTCAGTTCTCCAT	136
STMN4	125	AGCAGAGAAACGGGAACATGAGAGAG	CCTCCCTGTTCTCCTTGTGAGATT	102
TMM66	114	ACACCCTTCTCAGACTCGTGGTACT	TGAACATACCGAATAGCTGCCCGA	68
ADA28	104	TTTGCCCTGGAAAGGACGGATAGT	CACACTTAGTTCCATTGGCCACCA	102
UBXD6	95	TCTACTTCCTTTCCCAGACGGCCT	TCCTCCAGGATGAGTACAGTGCCA	102
PGFRL	87	TCCCAGTGGCCCTCCCTCAACAA	AGGACAGTGCAGAGCACACTGAT	102
ASAH1	80	AGCCTACTTTATCCTGGGAGGCAA	ATACATCCAATGATTCTTTCTGTCTCGTG	170
NM_199205	69	TGATGCCAACTGGTGTGGCTACTT	CGGATCCCAAGGGTAAGGAGGAT	205
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LZTS1	182	AGGAGAAGGAGAAGGTGATTCACTACC	AGTGGCTATGATGTCCTCGTAGGG	125
Q8N1G8	167	TACGGCCACCTTGATCTTGTGCAC	GGAGCACAGAGAGCTAGCATCAGG	63
ARHGA	153	GACCTGTCCTCCTCATCTGGGTCC	CGAGCTGGCCTTGGCTTTCTTGGC	63

LIPL	140	GTCTTACACACATTACCAGAGGGTC	TCTGCAATCACGCGGATAGCTTCT	250
Q8N8I7#	128	GGAGGAGATGTTGTGTGCATGTGAGA	TCTGTCTGCGTGAGCACATTCGCATA	125
BMP1	117	AGGTGTACTCGGCGGGAGATTCTGT	TGCTGTGGAGTGTGTCTGGAACCTT	63
Q16016	107	AGGCCTAATCGCCACTCATCAGCAAA	TCGGTTTGTTCAGAGGTAATGGAGGG	94
ENST356349#	98	AAGAGACTAGTGGCATGTTGTCCC	TGCTTAAAGATTCTTCCAACAGATAGCCA	313
Q9UDD8	90	TAGAAGGCATGCTCCTGACTGTGA	GGTGAGGATTCACGATGATTGGGT	63
ENST357748#	77	GAAGCTCACTGGTGTGTCTCCTCA	AAACATGGTGGCCAGCAGCATTTG	93
<u>ENST338711</u>	72	AAAGCATGGCCATCATCTCCACACA	ATTCTGCAATGTAGAGGGCGGCA	125
CLN8	68	GTCAGCAGCCTGTATCTGCCTCATTT	ATGATTAGCGTAAGCAGAGCCAGTCC	188
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PP2BC	168	AGGGTTCTCGCTTCAGCACAAAGAT	TTCTTCCCTTGGTCGCTCCTGT	156
Q86YR2	141	AGTCCCTATGCAAGTAGCCAGTT	GGAGGGAGGAATGATGATAAACCAGG	94
EFA6R	129	CAGCATTTCTCAAGGAAGGAGGCAA	CGCTTGACTTTGGCAGTGATTGGA	94
Q7L3Y3	118	CCCTGCAGAGAGTGGTGATAACACAT	GAAGTGGCATTTGAAACCCAGGCT	94
GFRA2	99	TCATCCCAGGGAGTAACAAGGTGA	GTTTCAGCATCAGGACAGACAGCA	250
MTMR7	91	CCGACAGTCAGTTACAGATTACCTAATGGC	ATGTCTTACTTCTTCCAGGGCCTC	219
Q8NB85	84	TGTCCTGTGAAACCCTGTTACCTC	AGTAGGAGGTGACTCCTTATGGGCA	188
SH24A	78	GAGGAACCCATCACTTCCCTGG	CAGGTAGTCAGGCAGCTGGT	250
ZDHC2	73	TGCAAAGCCATTGAGAGAGTCCCA	GTTTATGCTGCTCTCCGTCCAAGA	188
SPG11	65	AGGCTCTCGGAGAACTCAGGGAAA	GCGTAGCAGCTGAAACCCGTTTGT	156
ENST360191#	61	AGGAAGGCTCTCCTCCACATGTTT	TTCCATTGCGCAGCGGCTTCTCTG	94
13				
FGF17	217	TGTTACGGAGATCGTGCTGGAGAA	GAGCCCACAACTCGAACTGCTT	115
Q9NUU8#	200	CACTGCCCACCGCCACCTCA	GCTGCAGCACAAAGATCCTGGAGACT	58
SFRP1	169	CACCAGCTGGACAACTCAGCCAC	ACTTAAACACGGACTGAAAGGTGGG	58
PIWL2	142	CTGTGCCACATGTACTGGAATTGGCCT	ACAGGAAGAACAGGTTCTCGCACA	58
DOK2	130	ATATACGATGAGCCCAGGGAGT	CTGGCTGGACATGCTGGAGG	87
CGAT1	119	ATGAGAAGCGCTGCATGGACGA	TATCTCGTGCCTGAACACCAGCAT	87
Q8NEE2#	109	ACAAAGACACCCGGAACCACCACA	AGGGACCATATCCGGGAGAGC	288
CTR2	100	ACAATGAAGAAGATGCTTATCCAGACAACG	GGTGAAGTGAAGTTTCTTGGGTGATGG	115
Q71JB5	79	TGCTGGAGGAGTTCTGCAAGGA	GAGACGTTCTGGCGAGGATCATGT	87
VMAT1	74	TATGCAACCCAGAAGCCACGAA	TACTCCTCATGGTCAGGCTCCTCAT	87
DEMA	70	TCTCAAGGTATTTGCCATGTCCC	TTGAGCTCATTCGCTTCCACAGA	115
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	PHYIP	201	TGCATGTACACGGCCTACCACTA	GTCGACGGGCTCAGTGTAGATGAT	80
	GFRA2	170	AACGCCATCCAGGCCTTTGGCAAC	TGGACAGACGTGCAGGTGGTGATGA	107
	Q9P1G9	156	CAAGCCAGGGTTGGAAGAACCAAA	TGAGTCAATGGCCTTCACCTCCAT	80
	LGI3	143	GGGACGACAGAAGTTTGTACGGTT	CCACCACAATGTGTCTATACACCAGC	80
	PDLI2	131	CAGGCTGTGCGCATCCAGGAG	CGGGCATGCTTCTCACAGTACA	80
	INT10	120	CACCACACTGTAACCTCGAGGCAT	AATGCAGAACCTGTGCAGAACCAC	107
	BIN3	110	ACCTGTCCCATCAGCTTGACCA	TCAGTCATCGGCCACAATGGAGA	107
	Q96BB3	93	GGAAGGGCAGCATCTTGATTCCAT	GTCCTGAATACATGGTGAGGACCAC	107
	XPO7	86	ACAGTATTGTGAACAGCCAGCCAC	ATTCGCTCGATGCCTTCCATCAG	107
	PSPC	80	TGGAATGCTCTCTGCAGGCCAA	TGCTGAGCCTGCATCTCGCC	134
	NP_1013864	75	ACCTCCCTGAAAGGCTCAGT	CTCGTTGCTTCTTAACCACTAATGATGGCA	268
	RPO3D	71	CTGGGACACGTGAAGCACAAACTT	TACCGGTGTTTGTGATCCAAGAGGGA	107
	BD02	67	ACCTGCCTTAAGAGTGAGCCATA	TGCCAATTTGTTTATACCTTCTAGGGCA	80
	ENST357748#	62	AGCCTACACCATAATCTCAGGATCCACG	TGAGGAACACACACCAGTGAGCTT	161
15	NKX31	193	CTCACGGAGACCCAAGTGAAGATA	CCACGCAGTACAGGTATGGGTAGTAA	125
	TR10B	162	TAAAGGTGGCTAAAGCTGAGGCAG	AGTGGTCTCAATCTTCTGCTTGG	63
	STC1	148	CAGACAGACCACTGTGCCCCAAACA	CACTCTCATGGGATGTGCGTTTGA	94
	CHMP7	135	GACATCCTCCTTCAGGATACCACCAA	GGACAGTTTCTCAAGTTCAGCTTC	94
	Q9Y3T6	112	AACTCCTGCGTCTGGTGAAGG	CGGACAGCAGGCCGCTCTTCTTTT	94
	NFL	102	AAGAAGAAGGAGGTGAAGGTGAAG	TCTTCTTAGCTGCTTGTTCTCTCCC	156
	TR10D	93	CACTGGAAGAAGGACATGCAAAGGA	TAGCAGAGCCTGCCTCATCTTCTT	125
	MFRN1	85	TCCAGTCCATCCACTTCATCACCT	ATGATGTGGGACTGCGGGTTGTAG	94
	TR10A	78	CCTGCCTCTTCTCATTACCTCTCA	TCAAACAAACACAATCAGAAGCACA	94
	ADAM7	72	CACCATCTTGGTTGTTGTGCTTGTC	CGGTAACGAACTAATAGTATAAGAACTCCG	188
	PEBPL	67	TTCCACCTGGGCGAACCTGAA	GGGTGGTGAGTCCTGGTAGTTCT	125
	LOXL2	63	GGTTCCTTCAGCGAAGAGACGGAAA	CTGGTTGTTTAAAGAGCCCGCTGAAGT	125
16	SCAR3	191	TCAAAGGGCAGCTTTGGAAGTGA	ATCCCTGGTTCACCCCTTAGGC	94
	Q6P464	160	CCTGCTGCTGGTTCTTCCGAT	CAATCCTTTCCAAGGCAGTCAGAGAG	125
	TRI35	146	CGCCACTGCCACCTGTACACCTTC	CAGCCATCCAGTTCTTCTCTTGACA	125
	ENST355177	133	TCATCAGAAAGTACCACAGACTGGGC	TGCAAGCCAAATAGAGAGGCCTCAGA	63
	BNI3L	121	GAGTTCCACTTCAGACACCTTAAACG	GGAAGAGAGATGGAATGAACACCTTCAG	188

CLUS	110	AGCTCTTTGACTCTGATCCCATCAC	TTTGCGGTATTCTGCAGCGCTTT	125
FAK2	91	AGATGCTGACGGCTTCACACA	TGGGCCAGATTGGCCAGAACCTT	125
ADA1A	83	AGGTCTGCTGCTGTGTAGGG	GGAGATGGTGTGGACCTTAATGGTTG	94
Q7Z2R7	76	GGTCCTCTGTGTAGGTGAATGTGTCC	CACCTCTAACAAGACAGTCACACTGAAA	94
HMBX1	70	AGCAATCCTGGAGAGTCATGGGAT	GTCGACATCATCACTGTTTGAGTGGC	94
KCTD9	65	GTGATCTGTCTGGGTGTGATCTTCA	TATAGCTCCCTTCACGTGGACCTT	94
GON1	61	GTGCGTGGAAGGCTGCTCCA	TCTCTTTCCTCCAGGGCGCAGTCCATA	125
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DUS4	176	TTAAGCAGCGCCGCAGCATCATC	AAAGCTGAAGACGAACTGCGAGGT	75
Q96T53	147	CAAGAAAGTGAACCAAAGCACAGC	CCCAGCAAACGAAACCAAACACCT	113
NP_060720	122	TATTAAGGTGGAGGACACAGCCAAGG	GCACTCTGAGCATCTCGTCATTGT	150
TOPK	111	GGCCACCTATTAATATGGAAGAACTGGATG	CAATGTGTGCAGCAGAAGGACGAT	60
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DCTN6	92	TTGACAAGTGGCTGCATCATTGGG	AGGCAGTCTGCACCATAGATCACC	75
COE2	84	ACCGTTGGGTCTTCCAGCACAT	GGCAAAGGCACCTCTTCTGTTTGAC	113
HYES	71	ATCCTGATTCCGGCCCTGATGGT	ATGTGCTGGGACATCTGAGGAAC	150
PNOC	66	AATACTTGGTCCTGAGCATGCAGTCC	ACACATTACCATTCTGGTGCAGGG	225
DPYL2	62	GCACCACCCAGCGTATCGTG	CTAGCCCAGGCTGGTGATGTT	300
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NP_076930	148	ACTGGCCTACCTCATGCTGTACCA	TCATGCTTCCAGACCCTGCC	231
UNC5D	135	TATTTGCTACACAAAGTAGCCCATCTGC	GTTTGAGAGTTTCGTGTGTCTCTCC	144
EXTL3	123	GCATCAACTTCTTCGTGAAGGTGTACGG	AGATGAACTTGAAGCACTTGGTCTTGTC	144
WRN	112	TGCACTTATCCCAAGCGGTGAAAG	TTGACGGGAGGGTTTCGGATAACA	144
FAK2	102	AGTGAGGAGTGCAAGAGGCAGAT	ATTGGCCAGAACCTTGGCCT	231
LERL1	93	AGCTTGTGCACTTGTCTCACAGG	CAGCTGAAGTCGTCATTGCTTCCA	115
NRG1	85	AACACAAGCTCCCAGAGCAGTAAC	TCTGTATGCCCAGGAAAGGCGTAT	173
PP2AB	78	TTGGTGTGTCATGATCGGAATGTGGT	ATAGCAGCCTGGTTCACACAACGATA	202
Q7Z4A1	72	AAAGGAGAGAAAGGAGACAGAGCTGG	CAACACCATATCCACGGCCC	173
Q7Z2R7	67	TGTAGGTGAATGTGTCCCAAACCTGC	CACCTCTAACAAGACAGTCACACTGAAAC	202
ZN395	63	TCTGCCATCCTTCCAGATCCCAGT	GCAGCAGCCCAGCTGACACT	144
ENST332498	60	TTATGAAGTGTTCCAGTGCCACACT	TTGGCCCAATCTTGTGCTTTCCTC	144
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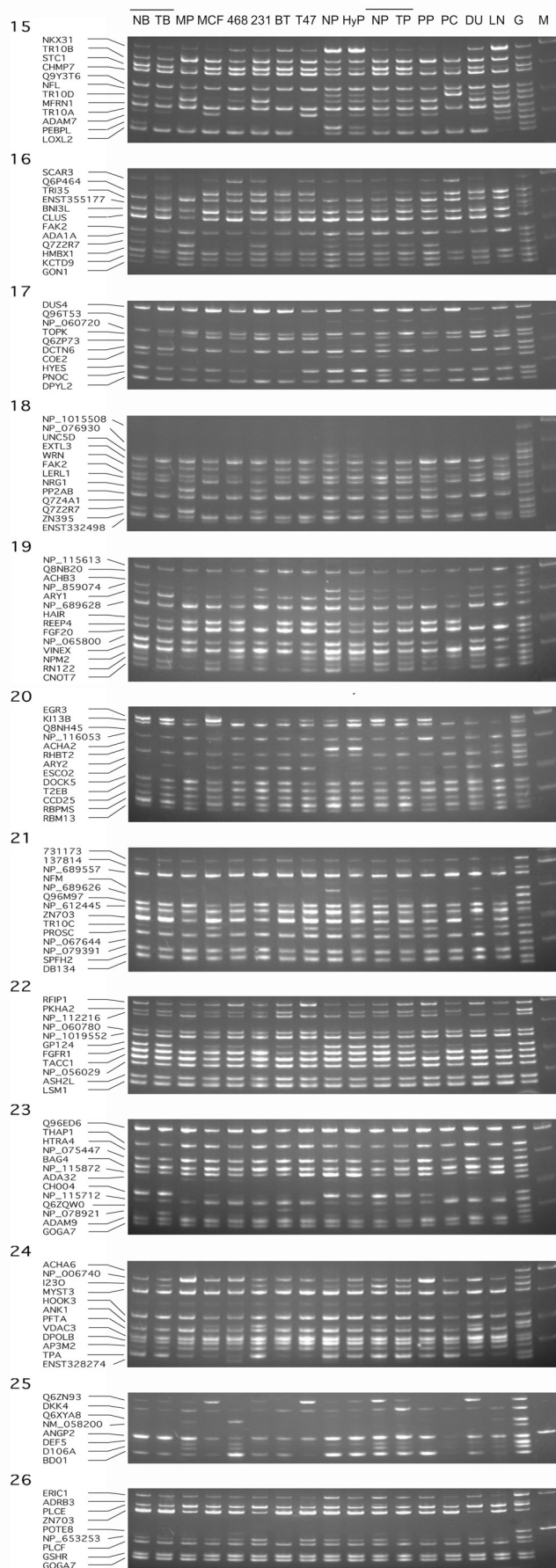
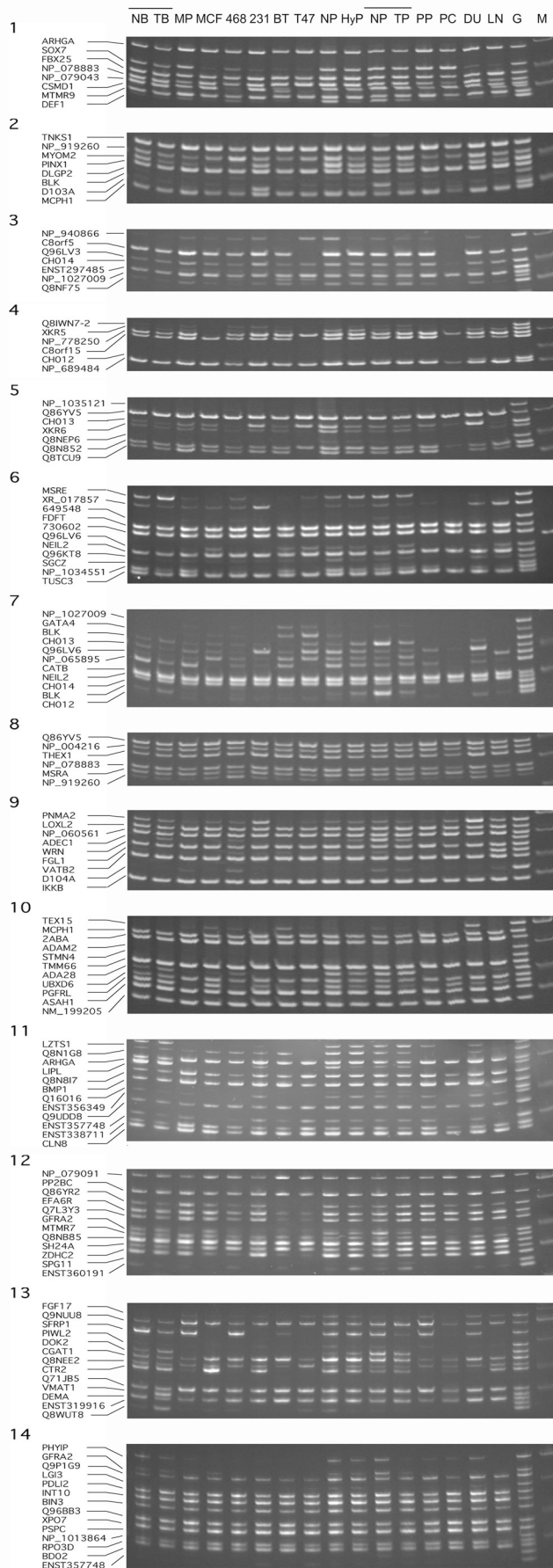
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HAIR	106	ACAGTCAGCGTCACTCAGCACTT	CATAAAGCAGGTGGCAGTCAGGG	161
REEP4	97	AGTGTGGTCAGATACTGAGGCAG	TTCTCTTGACCACACGCAGGCT	161
FGF20	89	TTCCGAATGCATCTTTAGGGAGCAG	TGCGGCCAGTGTCTCCATGTTTAT	107
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VINEX	76	CCAGAACGAAGACGAGCTGGAG	AAACCAGCCATCGTCACACTGCT	214
NPM2	71	GAGGAAGAGGAAGATGATGAGGATGAGG	ACTTGTTTGACAGGGCTTTGCTCC	161
RN122	67	TCTGGTGAAATGGCTGGAAGTTCG	TGAGGGACTAGCAATGGGCTTGTT	134
CNOT7	63	ACAGGAGGTGGCAGAACAGTTAGA	TCAGATCCTGCCTGATGTTGTGGT	214
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EGR3	194	GGTGACGTGGAGGCCATGTATC	TGGTGGTAGAGGTTGTAGTCAGGA	144
KI13B	178	CTGACGTGCTGGTGACAGCAT	ACGCTGTGGGAGAAGTACCCGCTA	115
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NP_116053	149	TACGAAACAAAGACCTCCCTCAGC	TGGCCTCCAGAACTTCTCAGTGAT	87
ACHA2	136	GTTTGACAGTGACCCACATGACCAA	CATCTTGACAGTTCTGCTGGTCGAA	87
RHBT2	124	GTGGTGTTTCCCTACACAAGCAAG	AGAGGCGGTTGGCTAGAATGATGA	87
ARY2	113	GGGTTTACTGTTTGGTGGGCTTCA	CAGCACTTCTTCAACCTCTTCCTC	87
ESCO2	103	CCTAGGGCTTGCCAATGTTTCAGAT	GTCGTCTTGCAATGCGCTTTCTTC	87
DOCK5	86	AGCACAAAGGCCAAAGAGTCTCCA	TCAAGGGTGTTGACTGAGGAGGTGAA	87
T2EB	79	TTAGATCAGCATGACCAGCGAGGA	CTTTCTGGGAATGGGCAGTGCTT	87
CCD25	73	AAAGACCAAAGTCGAGCGGTTCC	CTCATTCCTCTCTTCACGATCTCTGC	115
RBPM5	68	TACCCTCTGTACCCAGCGGAGTTA	AGTGAAGCGGGATAGGTGAAAGCA	346
RBM13	64	ATGGCGACATCTACAACCTCCC	TCTGCCTCCTGTTGTTCCAGGG	144
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731173	208	AGGTGATGATGACCAAACGGACCA	ACTTTCTTGGCTTCCCATTCAGCC	107
137814	191	CTACAAATGCAAGAGACAGCGCCA	TGTAGCCTCCGTAGCAAGAGTAGG	268
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TR10C	91	TCCTGCACCATGACCAGAGACACA	TGCTACACTTCCGGGCACATCTCT	80
PROSC	83	ACCATAGCCATCGTGGAGCACATA	TGCCCCAAGCTTCCTATGGTCATC	107
NP_067644	76	TGGAAGTGGAGCAGAAGATGCTCA	GAGGAGGAATTTCTGCCAATGAGTG	134
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SPFH2	65	AAAGCTTCTCATTGCCGCCAGAA	TCTTCGCTCTGTCTCTGCTTCCTTT	134
DB134	61	CTGCAGACTTGAATGCTATGAGAGTG	CTCCAGCTGAAACATACAGTAGGC	214
22				
RFIP1	192	GCTCAAGCTTGGATAAACAGCTGCC	TGCTGGTGTGGTGAGTGTGAGAAT	68
PKHA2	176	TTTCAAGTGGGCCCACTCTATCC	AGGCGTGAACAAGGAGTCCTCTGAA	205
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GP124	111	TATCCACGCTGCTCTGGATGGG	TAGGACTGGGAGTAGGCAGAGC	205
FGFR1	101	TACATGATGATGCGGGACTGCT	TTGGAGGTCAAGGCCACGATGC	205
TACC1	92	GCCAATGAAGAGATTGCTCAGGTTTCG	TCCACCTTCATCTGCTCTTTGCG	205
NP_056029	84	CAGAAGAGACCTCTGTGGCAGTTA	CATAGTCAATGCGTTGGCCTCCAT	341
ASH2L	71	TGGTGTCAATCAAGGTGTGGCT	TGTACAGTGAGATGGCTGGGAAGT	341
LSM1	66	GCTGGAAGCAGAGAAGTTGAAAGTGC	TCTGCTCGAGGAATGGAAGACCT	273
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THAP1	167	ATCTTCTGGAGCCACAGGAACAGCTT	TTTCCGCTGGTGCATTGTATCCTC	115
HTRA4	153	CTCTGGATTGAGAGATCACGATGTA	GTTTCAGGTATGACTGTCAGGAGC	346
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BAG4	128	AATCAGGACCGACTGTACGACCACAA	TTCACTCATGTAGAGATTGCCGGG	144
NP_115872	117	CCATGATTGGAATGACATTTGCCCT	GCTTCTGTGCAGTGGAAGTACAAG	288
ADA32	107	ACACCTGGCTTCTAGGTTTCTCTCA	TTCTCTTCTCTGGCGAACCCTT	144
CH004	90	ACAAGAAAGCAGAGGAGAGAGCCA	TCTTCTTCAAGGCCATCAGGGCA	173
NP_115712	83	GATTGACCTGGAGAAGGGCTCACA	TTAGCTGGACAGCTCCAGATGCAA	144
Q6ZQW0	77	TGAGCTGCCAGTTCTGAAGGGT	ATAACCCATGGTGAGGAAGCTCAG	144
NP_078921	72	TCAGACAGATACTGTGGGCTCTGTG	GCCATCATAATGTGCTGGGCCAT	115
ADAM9	68	CCACAACCGAAAGTATCATCTCAGGG	TATAAAGGAGGTGCAGGAGCAGGA	231
GOGA7	64	GAAGAAAGTCTCCAAATACATTCAAGAGC	GAGGAGGCCTTGTGGAGCATAGAT	288
24				
ACHA6	199	TGATGAGGTGGCCTCTGGACAA	ACCACCCACTGTAATGGCTGATGA	250
NP_006740	183	CATGACCCATGGCTCTGTGAAATC	TAGTTGCTTTCTGGGCTGGCTT	375

I230	168	AGTCAAATCCCTCAGTCCGTGAGT	TCTTATTCTCCTTTGGCTGCTGGC	156
MYST3	154	TTAGCTGGGACTCCTCAAGCACAA	CTGGCATTGCCCCTTGCAATCTCT	156
HOOK3	108	AGACCTTGGTGATTTAAGGCGCA	GCTGCGTTGGCCTTTCTTAACCT	94
ANK1	99	TGCAGACGGCTCGATTGTCTCATA	GTACTTGTTCCTGGAATGAGTGTGG	125
PFTA	91	CGTGGTCTTTCCAAATATCCTAATCTG	CCACAAGAAAGGCAATTAGGTAGGG	313
VDAC3	84	ATTGACTTTATCAGCTTTAATCGATGGG	TAAGCTTCCAGTTCAAATCCCAAGCC	375
DPOLB	78	CTGTTACATCAGGTTGTGGAGCAGT	GAACCTTGTCTCACCTTTGACAG	313
AP3M2	73	GAGTCTTCAGGCTGGAGCTTCCAAA	CAGCTGCTGGATCTTAAACTGC	188
TPA	69	GCATGACTTTGGTGGGCATCATCA	TGTACACACCCGGGACATCCTTCT	156
ENST328274	65	GTCAATGTGATCCTGGTTATGCTCCTCC	ACTTCCTCCTGGTGATGACATTGC	313
25				
Q6ZN93	179	TTCGCTCTGGGACTCACTCTGAAA	TGTGGTCGTTTGGAGTCTCCTGAT	94
DKK4	150	TTTGTACTIONGATGGAAGATGCAACCC	CCTTCCTGCCTTGTGATTTCTT	188
Q6XYA8	125	CAGTGAGAATTGGATAGCTCCATCAGGG	CCATAGAGCAACTCTGCGAGAGAA	281
NM_058200	114	AGACATGTGAACCACTCAGCCACT	TAAGAGGTCCCGTTTCACTGCGT	188
ANGP2	95	CACCTTAAAGGACTTACAGGGACAGC	CATTTGTGCTTGTCTCCATCCTTTGTGC	141
DEF5	87	AAGAGCTGATGAGGCTACAACCCA	GCAGAGAGTCCATTTCTGCAAAG	188
D106A	80	AGGGACATGCAAGAACAATTGCGG	TGGTCCGACAGCATTTAGAGACT	94
BD01	74	AGCAGTGGAGGGCAATGTCTCTAT	TTCCCTCTGTAACAGGTGCCTTGA	281
26				
ERIC1	195	AGAACAAGCAGAATTAGAGAAACAGCAGAG	CTGCTGTCCTCGGGCTGGTACAT	250
ADRB3	179	TGGTCCTGGTGTGGGTGCTGTC	AAGAGGAAGGTAGAAGGAGACGGA	167
PLCE	164	TTATGAGTCACCAGATCCAGAAAGA	TCCAGGTGTTACATACAGCTTCC	417
ZN703	150	CTATGGCAAGAGCCACTTATCCAC	TGGAGGAAGAGCTGTAGTTACTGG	417
POTE8	95	ACTTGCTGTACGTTGTGGATCAGC	CAGCAGTTTGTCCAAATACATCTTGAG	417
NP_653253	87	TGAGTGTATCAGAACTACAGGCTGC	TGAGCTGTGTGCGAGTTGTTCT	167
PLCF	80	GGTGAAGGACACGTTCAAGGAGGA	AGCGGCTCCTGTCCTTGTGGTT	250
GSHR	65	ATGCAGGGACTTGGGTGTGATGAA	GTTGCTCCCATCTTCACTGCAACA	208
GOGA7	61	GAAGATCTATGCTCCACAAGGCCTCC	AACTCGCAGTCCTCGCTCAATA	292

† The gene names are abbreviated. Most names have "_HUMAN" at the end. The "00000" is removed from the numbers of the ENST names and "00" is omitted from the numbers of the NP-genes with 7 digits.

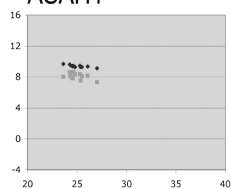
‡ These genes have been delisted from the Ensembl Database in Version 43.



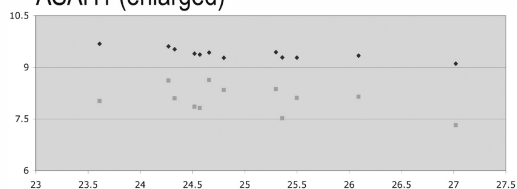
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ENSG00000136574	GAT4	chr23	11599122	11594920	7	27	81	104	70	41	282	757	28	39	43	39	65	409	-13	-8	-14	-6	-4	4	80	NP	PP	PC	LN	
ENSG00000191615	NP_001027009.1	chr23	11599122	11594920	7	27	81	104	70	41	282	757	28	39	43	39	65	409	-13	-8	-14	-6	-4	4	80	NP	PP	PC	LN	
ENSG00000154326	NEIL2	chr23	11694657	11692263	7	558	554	81	65	92	723	842	875	761	746	956	979	941	302	143	193	282	193	99	177	222	137	151	280	449
ENSG00000179007	730802	chr23	11684020	11685306	6	933	755	895	1043	1009	965	1005	1026	899	952	1266	965	591	2737	1622	1348	7897	3438	2427	4259	3414	2864	1166	5200	3887
ENSG00000079459	PDF1T	chr23	11697573	11734227	7	917	850	1035	1272	1183	1233	1155	1167	899	1081	1447	1211	440	2243	2158	769	1083	1968	328	1521	1383	3477	569	1649	529
ENSG00000164733	CATB	chr23	11698210	11698260	7	1537	1518	1697	1822	1743	1671	1696	1267	1656	1132	1545	1483													
ENSG00000177586		chr23	11823510	11824424																										
ENSG00000102107		chr23	11830789	11830960																										
ENSG00000225884	DB136 HUMAN	chr23	11868873	11869117																										
ENSG00000225883	DB135 HUMAN	chr23	11877239	11879472																										
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ENSG00000185945	NP_958804.1	chr23	12032086	12033678																										
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ENSG00000225879	NP_116305.2	chr23	12071227	12070931																										
ENSG00000197246	653726	chr23	12077613	12077915																										
ENSG00000186523	NP_116305.2	chr23	12078249	12088988																										
ENSG00000225877	389633	chr23	12161408	12319912																										
ENSG00000184352	653726	chr23	12327008	12327160																										
ENSG00000145002	FAM69T	chr23	12327494	12338223																										
ENSG00000225874	QSOX3 HUMAN	chr23	12378422	12379002																										
ENSG00000225873	QSOX3P HUMAN	chr23	12382711	12468021																										
ENSG00000197203	QSOX3 HUMAN	chr23	12480186	12483169																										
ENSG00000177400	QSOX4 HUMAN	chr23	12586140	12588894	20	17	36	36	46	36	23	43	20	42	40	26	75	342	-1	4	-2	-4	-6	4	9	6	-7	16	-4	-1
ENSG00000201022	NP_001034551.1	chr23	12623790	12624008	4	852	876	777	440	850	659	707	912	824	234	713	977	717	238	245	174	98	251	94	96	342	248	32	177	510
ENSG00000154359	NP_001034551.1	chr23	12623790	12624008	4	852	876	777	440	850	659	707	912	824	234	713	977	717	238	245	174	98	251	94	96	342	248	32	177	510
ENSG00000173571	NP_001034551.1	chr23	12623790	12624008	4	852	876	777	440	850	659	707	912	824	234	713	977	717	238	245	174	98	251	94	96	342	248	32	177	510
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ENSG00000225899	NP_001034551.1	chr23	12623790	12624008	4	852	876	777	440	850	659	707	912	824	234	713	977	717	238	245	174	98	251	94	96	342	248	32	177	510
ENSG00000164741	NP_079043	chr23	12685243	13147656	1	553	375	480	24	648	124	7	254	242	394	524	394	394	187	120	56	31	162	47	10	50	34	3	26	186
ENSG00000200630	Y	chr23	13062058	13062157																										
ENSG00000164743	XR_017857.1	chr23	13486723	13470167	6	149	47	94	115	520	26	40	169	31	29	338	596	566	70	46	81	33	113	74	56	31	57	57	102	91
ENSG00000186533	Y	chr23	13486723	13470167	6	149	47	94	115	520	26	40	169	31	29	338	596	566	70	46	81	33	113	74	56	31	57	57	102	91
ENSG00000199611	Y	chr23	14221336	14221396																										
ENSG00000206950	Y	chr23	14234435	14234541																										
ENSG00000199127	hsa-mir-383	chr23	14755318	14755390																										
ENSG00000104723	649548	chr23	15845005	15845412	6	477	471	678	754	702	626	732	610	692	992	769	570	340	509	1232	379	620	804	480	636	1022	1123	339	1091	319
ENSG00000147333	MSRE	chr23	16006761	16004596	6	398	42	32	128	44	56	86	615	23	28	70	40	572	-3	-28	-28	-14	-15	-10	-12	-35	-29	-22	-31	-21
ENSG00000078579	PGF20	chr23	16804409	16804061	19	5	22	23	13	29	9	12	24	13	16	15	7	189	-15	-22	-16	-9	-4	-27	-27	-19	-23	-21	-29	-20
ENSG00000155970	NP_069074	chr23	17036383	17036383	19	436	807	608	243	355	20	43	81	41	14	26	124	193	14	12	22	23	30	62	625	31	16	-16	-14	11
ENSG00000104219	ZDR2C	chr23	17066667	17122305	12	594	633	206	110	851	115	60	651	533	701	863	801	408	514	403	471	424	550	344	625	452	341	206	841	820
ENSG00000198791	CNO7T	chr23	17131108	17148758	19	271	116	335	87	276	172	189	143	168	139	344	490	367	-3	3	-5	9	11	0	22	9	2	-1	-1	-3
ENSG00000155975	NP_689628	chr23	17148851	17197438	19	594	1133	700	497	1525	1015	1056	539	633	240	777	994	292	-5	-13	25	-4	-2	-6	-20	-7	0	-16	-9	-13
ENSG00000203989	CTH2	chr23	17440585	1742296	13	747	69	2550	50	1241	51	41	201	24	75	58	528	483	-6	-13	25	-4	-2	-6	-20	-7	0	-16	-9	-13
ENSG00000203989	CTH2	chr23	17440585	1742296	13	747	69	2550	50	1241	51	41	201	24	75	58	528	483	-6	-13	25	-4	-2	-6	-20	-7	0	-16	-9	-13
ENSG00000104213	PGRLH	chr23	17478986	17544896	10	570	29	516	58	243	73	112	312	14	362	178	184	400	1702	2	421	30	107	9	31	154	8	180	84	138
ENSG00000124422	NP_058500	chr23	17545584	17702666	19	1039	756	262	625	582	723	507	565	313	81	515	455	175	706	380	89	343	140	200	154	652	324	15	336	282
ENSG00000210287	FGL1	chr23	17661599	17797693	9	61	22	24	66	23	61	25	72	16	30	30	584	424	7	-3	-3	9	-10	-4	-15	1203	961	261	1192	899
ENSG00000078674	QSOX3	chr23	17824788	17929534	12	992	1249	1152	906	1009	1189	111	1071	965	676	1024	980	310	903	2247	682	546	743	468	723	397	231	160	226	325
ENSG00000104763	ASAH1	chr23	17958414	17966757	10	695	624	122	648	735	821	780	689	673	551	662	620	449	1320	184	276	283	274	259	392	397	231	160	226	325
ENSG00000171428	ARY1	chr23	18164483	18164552	19	139	287	628	149	339	371	599	101	46	54	182	205	141	1123	19	99	90	136	137	261	235	26	94	222	196
ENSG00000210291	ARY2	chr23	18230335	18302962	20	17	36	19	50	22	47	18	32	20	29	14	27	410	43	8	-14	11	23	3	16	5	4	12	27	35
ENSG00000181897	Q7L3T3 HUMAN	chr23	18430276	18430550	12	347	645	452	163	460	48	53	303	462	477	579	517	358	206	403	115	24	266	7						

Ensembl		External		Chr	Start	End	Set	SM RT-PCR Results										G	Illumina DNA Microarray Hybridization Results											
Gene ID	Gene ID	Band	Pos (bp)					Pos (bp)	End (bp)	NP	PP	PC	DU	LN	NP	PP	PC		DU	LN	NP	PP	PC	DU	LN					
ENSG0000011320	ESCCO2	p21.1	2768759	2771881	20	15	28	208	211	565	485	402	187	83	148	318	901	378	3	-11	12	5	-1	-20	21	-13	-5	3	31	33
ENSG00000168078	TCPK	p21.1	2772356	2775135	17	16	167	513	224	789	618	648	271	125	407	848	696	339	21	129	49	36	986	394	1183	26	157	518	1050	1846
ENSG00000168079	Q7Z4A1	p21.1	2778372	2780517	17	25	18	20	9	12	12	20	68	56	86	28	18	411	4	11	-9	-2	-3	8	-1	-2	11	5	-2	-8
ENSG00000188233	OC2P73 HUMAN	p21.1	27935807	27997307	17	59	32	29	30	24	24	20	309	22	24	15	50	461												
ENSG00000146031	NP_005651	p21.1	2800328	28014592	9	563	566	646	482	538	456	525	474	579	657	594	643	468	737	395	245	367	352	204	425	502	517	643	346	181
ENSG00000188081	PNOC	p21.1	2823258	28256786	17	19	18	21	8	14	14	20	359	50	62	34	29	417	737	395	245	367	352	204	425	502	517	643	346	181
ENSG00000188079	ZNF653	p21.1	2823258	28256786	17	18	832	603	671	851	721	710	714	747	830	1034	951	650	397	1580	456	956	931	843	276	831	1502	861	742	734
ENSG00000202761	U6	p21.1	28256884	28283967	17	59	32	29	30	24	24	20	309	22	24	15	50	461												
ENSG00000104290	F2D3	p21.1	28407692	28487707	17	59	32	29	30	24	24	20	309	22	24	15	50	461	155	177	194	108	94	46	106	406	185	46	126	202
ENSG00000202841	SS RNA	p21.1	28443847	28443955	17	466	953	515	1057	936	775	1010	810	1605	1552	1257	888	537	140	257	93	291	203	48	98	110	392	188	187	135
ENSG00000102922	EV13	p21.1	28688109	28688338	17	372	739	406	373	453	347	440	590	386	551	807	387	450	315	393	212	381	183	113	238	195	238	383	396	256
ENSG00000210500	NP_060720.1	p21.1	28685886	28689593	17	286	588	686	686	686	686	686	286	588	686	686	686	286	588	686	686	686	686	686	686	286	588	686	686	686
ENSG00000210509		p21.1	28685886	28689593	17	286	588	686	686	686	686	686	286	588	686	686	686	286	588	686	686	686	686	686	686	286	588	686	686	686
ENSG00000210510		p21.1	28685886	28689593	17	286	588	686	686	686	686	686	286	588	686	686	686	286	588	686	686	686	686	686	686	286	588	686	686	686
ENSG00000210511	HMBY1	p21.1	28685886	28689593	17	286	588	686	686	686	686	686	286	588	686	686	686	286	588	686	686	686	686	686	686	286	588	686	686	686
ENSG00000202079	SS RNA	p12	28696136	28696204	17	356	512	379	283	301	371	348	353	607	268	232	555	547	321	812	221	20	105	177	194	246	826	94	156	87
ENSG00000187992	K1R38	p12	28698075	28719550	20	653	422	386	818	900	571	441	706	655	396	232	113	331	375	293	203	443	254	134	389	705	225	176	164	31
ENSG00000120875	DUS4	p12	28249539	28264104	17	1232	1241	1693	775	1652	1435	549	1228	653	1607	319	581	564	2759	849	3598	904	3567	1011	497	282	570	317	150	267
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ENSG00000133872	TMM65	p12	30040184	30060201	17	787	761	815	783	876	819	833	710	766	754	767	728	489	4285	3344	1953	1157	2433	1598	2507	8975	3304	1129	2443	3453
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ENSG00000177699	G9F753 HUMAN	p12	30150001	30105950	17	34	162	34	24	34	25	25	88	97	25	66	39	414	563	552	562	320	746	394	428	465	482	202	649	10
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ENSG00000197285	T2B	p12	30355422	30353260	17	86	408	562	489	1087	592	495	713	859	781	675	1054	282	861	987	1288	865	1740	632	938	796	1348	1186	1986	1247
ENSG00000120139	U6	p12	30370448	30370448	17	559	429	641	731	538	753	534	642	456	566	613	745	401	248	136	585	583	371	944	372	701	197	553	1610	1184
ENSG00000202052		p12	30708725	30708724	17	213	417	516	270	458	385	505	294	362	350	556	525	392	105	185	238	64	141	84	128	89	121	134	313	395
ENSG00000104981	UBIYD5	p12	30712124	30744054	10	724	704	688	834	900	1174	1038	1086	947	840	1116	941	264	1309	1851	728	1135	1343	881	754	1445	1452	681	1589	884
ENSG00000120875	PEY45	p12	30868604	30820675	10	33	41	101	37	59	215	31	43	23	21	346	21	376	6	3	9	8	-15	25	11	3	11	6	144	84
ENSG00000172733	NP_1015508	p12	30972863	31010773	18	26	46	43	49	54	35	41	60	103	252	74	54	404	50	103	252	74	54	404	50	103	252	74	54	404
ENSG00000185392	WRN	p12	31013020	31150618	9	472	631	602	336	559	548	500	426	632	371	576	569	450	136	624	189	127	188	182	158	144	309	146	295	305
ENSG00000202141	SS RNA	p12	31571518	31575261	17	137	289	318	285	341	341	341	237	298	285	285	341	169												
ENSG00000157168	NRG1	p12	31617043	32741608	18	143	789	26	14	372	27	11	212	497	203	58	63	269	18	358	23	11	390	4	-5	16	130	7	20	10
ENSG00000210547	SS RNA	p12	32189125	32186230	17	223	235	235	235	235	235	235	223	235	235	235	235	223	235	235	235	235	235	235	235	235	235	235	235	235
ENSG00000202046	SS RNA	p12	32233554	32233681	17	325	325	325	325	325	325	325	325	325	325	325	325	325	325	325	325	325	325	325	325	325	325	325	325	325
ENSG00000198238		p12	32257645	32257948	17	305	1014	36	69	545	25	47	357	562	30	123	78	443												
ENSG00000187508	Q7Z2R7 HUMAN	p12	32743185	32744926	18	213	417	516	270	458	385	505	294	362	350	556	525	392	105	185	238	64	141	84	128	89	121	134	313	395
ENSG00000210559		p12	32782949	32783015	17	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232
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ENSG00000210569		p12	32985062	32985062	17	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232
ENSG00000210572		p12	32989476	32989454	17	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232
ENSG00000210574		p12	32991072	32991138	17	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232
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ENSG00000210582		p12	32992188	32992250	17	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232	232
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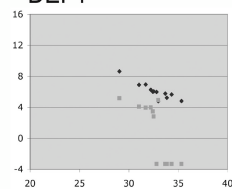
ASAH1



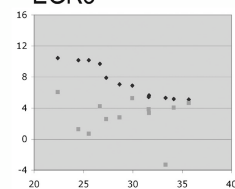
ASAH1 (enlarged)



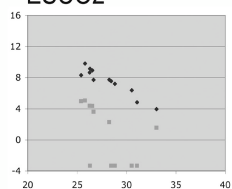
DEF1



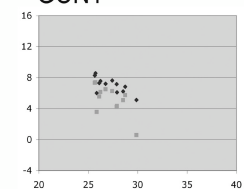
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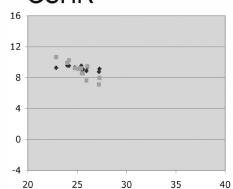
ESCO2



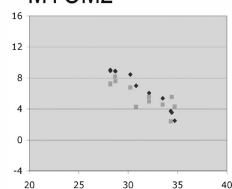
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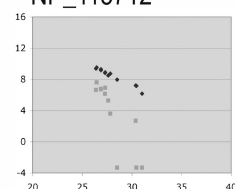
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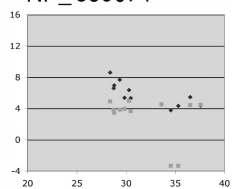
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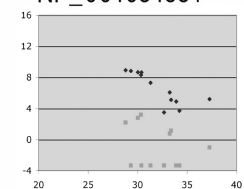
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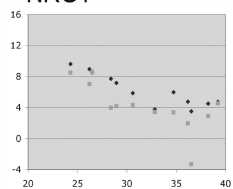
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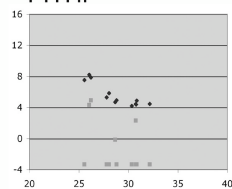
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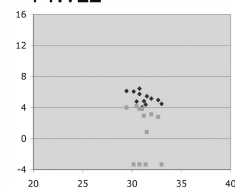
NRG1



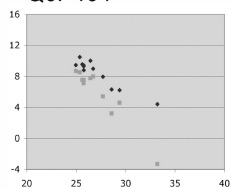
PHYIP



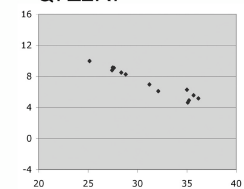
PIWL2



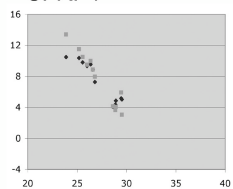
Q6P464



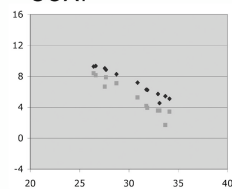
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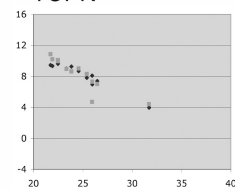
SFRP1



SOX7

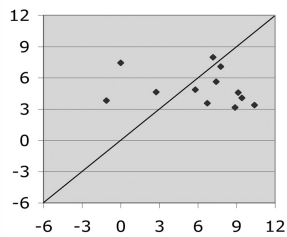


TOPK

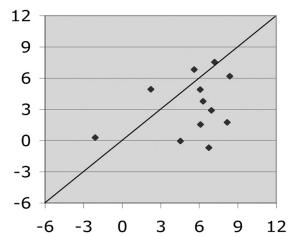


Up-regulation Genes

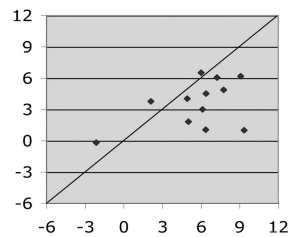
ESCO2



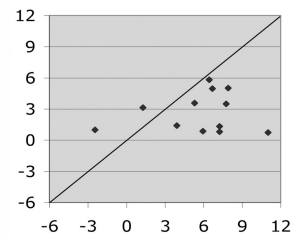
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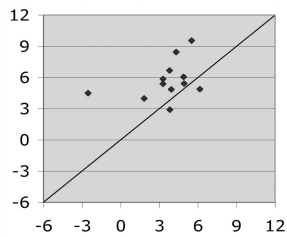


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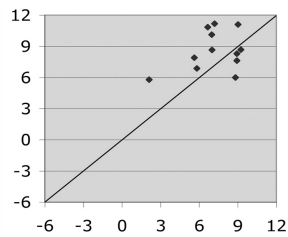


Down-regulation Genes

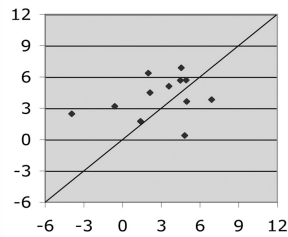
MYOM2



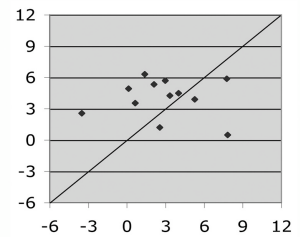
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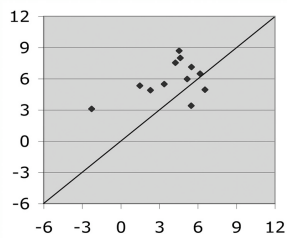
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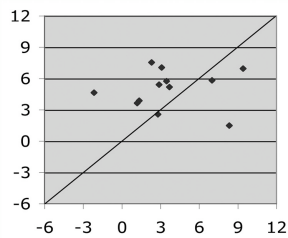
NRG1



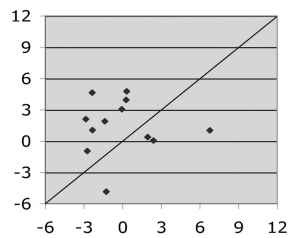
PHYIP



Q7Z2R7



SFRP1



SOX7

