Development of an angiogenesis-focused cDNA chip and validation of its functionality

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Abbreviation: bFGF, basic fibroblast growth factor; Flt1, fms-related tyrosine kinase 1; HDAC, histone deacetylase; HIF-1 α , hypoxia inducible factor-1 α ; INK, cyclin dependent kinase inhibitor; MMP, matrix metalloproteinase; STAT3, signal transducer and activator of transcription 3; TSA, trichostatin A; TSP, thrombospondin; UPAR, human urokinase-type plasminogen activator receptor; VHL, von Hippel-Lindau

Abstract

DNA chip has been used as a powerful tool to study the genetic reprogramming of cells and its link to cellular phenotype such as angiogenesis. To evaluate the angiogenesis related genetic reprogramming more efficiently, we here developed an angiogenesis-focused cDNA chip containing 153 angiogenesis related genes arrayed in duplicate on a slide glass. In order to validate the functionality of the angiogenesis-focused cDNA chip, we examined gene expression profiles in HT1080 cells treated with either fetal bovine serum, a well known pro-angiogenic factor, or trichostatin A, a known angiogenesis inhibitor, using the cDNA chip. All duplicate data from the analysis are well matched with each other and gene expression profiles are well consistent with

previously reported data. These results demonstrate that the angiogenesis-focused cDNA chip developed here can be a useful tool towards angiogenesis-related researches.

Keywords: angiogenesis; DNA chip; duplicate spots, gene expression profile; histone deacetylase

Introduction

Angiogenesis is an important physiological event known to be related with rheumatoid arthritis, psoriasis, diabetic retinopathy, artery stiffening and inflammation, as well as cancer (Folkman, 1971; Folkman, 1972; Rand, 1981; Colville-Nash and Scott, 1992). A number of genes are known to be involved in the event, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and matrix metalloproteinases as proangiogenic factors (Schweigerer *et al.*, 1987; Connolly *et al.*, 1989; Haas and Madri, 1999) and von Hippel-Lindau (VHL), thrombospondins, and tissue inhibitor of metalloproteinases (TIMPs) as antiangiogenic factors (Taroletti *et al.*, 1990; Moses and Langer, 1991; Maxwell *et al.*, 1999). Despite the importance of angiogenesis, researchers have found difficulty to study on a variety of genes related with angiogenesis in a high content way, because DNA chips focusing for angiogenesis have not been developed. DNA chip (microarray) technology has been highlighted as one of powerful ways of genomics study (Shalon *et al.*, 1996). Many fields, including drug discovery and toxicological research, have been benefited from the use of DNA chip technology (Butte, 2002; Kim *et al.*, 2003). However, some short comes in DNA chip technology such as low data reproducibility have to be overcome for better application to genomics study (Kothapalli *et al.*, 2002).

We here report the development of an angiogenesis-focused cDNA chip using PCR products of about 153 angiogenesis-related genes, which consists of two identical blocks. Duplicated spots in the cDNA chip could provide more reliable data than those of conventional DNA chip leading to save the time and cost of the DNA chip experiments. Expression profiles of angiogenesis-focused cDNA chip with human HT1080 fibrosarcoma cells treated with angiogenesis inhibitor/activator were investigated and validated the usefulness of angiogenesis-focused

cDNA chip for angiogenesis related researches.

Materials and Methods

Polymerase chain reaction (PCR) of angiogenesis-related genes

PCR primers for amplification of 153 angiogenesis-related genes were designed as follow; 1) poly (A) upstream and Alu sequence were eliminated, 2) sequence homology of PCR target regions was minimized, 3) amplicons were 0.2-1 Kb in size. DNA fragments were amplified from 10 human cDNA Libraries, validated by sequencing, and prepared over 7.2 µg per fragment.

Fabrication of angiogenesis cDNA chip

DNA fragments were spotted duplicate in two blocks by 417 arrayer (Affymetix, Santa Clara, CA) and post-processed according to the previous report (Skena *et al.*, 1995). Following post-processing, quality controls (QCs) were performed through 3 steps; non-specific hybridization QC, position hybridization QC, and surface QC. QC-passed angiogenesis DNA chips were stored in a dark dessicator and used for further analysis.

Expression profiling of HT1080 cells

Human HT1080 fibrosarcoma cells were treated with either 10% fetal bovine serum (FBS) as angiogenesis activator, or with trichostatin A (TSA) in 10% FBS as angiogenesis inhibitor for 12 h following serum starvation for 12 h. HT1080 cells were harvested and total RNAs were isolated. RNA from test groups was labeled with Cy5-dUTP by reverse-transcription and RNA from control groups with Cy3-dUTP. Labeled products were set onto the angiogenesis cDNA chip and incubated at 65°C for 16 h in a dark water-bath. Following wash, scanning by 428 scanner (Affymetrix, Santa Clara, CA), raw data extraction, and statistical analysis using GeneSight data analysis software ver. 3.5 (BioDiscovery, Los Angeles, CA) were achieved.

Signature correlation coefficient value (ρ)

Correlation coefficients between two signatures were calculated using the equation:

$$\rho = \frac{\sum_k x_k y_k}{(\sum_k x_k^2 \sum_k y_k^2)^{1/2}}$$

where x_k is the $\log_{10}$ of the expression ratio for the k^{th} gene in the x signature, and y_k is the $\log_{10}$ of the expression ratio for the k^{th} gene in the y signature (Marton *et al.*, 1998).

Table 1. Gene list of Angio_Chip

Category	Total number	Examples
Angiogenesis activator	87 genes	VEGFs, MMPs, HIF-1α, <i>etc.</i>
Angiogenesis inhibitor	26 genes	VHL, TIMPs, TSPs, <i>etc.</i>
Oncogene	14 genes	<i>c-Myc</i> , <i>c-Jun</i> , <i>ErbB2</i> , <i>etc.</i>
Tumor suppressor	20 genes	p53, INKs, APC, <i>etc.</i>
Internal control	6 genes	tubulin, actin, GAPDH, <i>etc.</i>

Results

To fabricate angiogenesis-focused cDNA chip (designated as Angio_Chip), we first selected 153 angiogenesis-related genes from GeneBank database (<http://www.ncbi.nlm.nih.gov>) and Angiogenesis database (<http://angiodb.snu.ac.kr>). The selected genes were amplified by PCR from 10 human cDNA libraries, including bone marrow, liver, placenta, brain amygdale, brain caudate nucleus, brain cerebellum, brain corpus callosum, brain hippocampus, brain substantia nigra, and brain thalamus (Table 1). The amplified cDNAs were validated through sequencing analysis and were spotted in duplicate onto glass slide by Affymetrix 417 arrayer. Quality controls (QC) of the cDNA chip were performed through 3 steps as described in Materials and Methods.

cDNA chip analysis was performed through the experimental conditions shown in Figure 1. Human fibrosarcoma HT1080 cells were used for this study, because the tumor cell line highly expresses angiogenic factors, such as matrix metalloproteinases and growth factors in response to various stimuli (Morodomi *et al.*, 1992; Mori *et al.*, 1999). These angiogenic factors are essential for the activation of endothelial cells during angiogenesis (Connolly *et al.*, 1989; Haas and Madri, 1999). Two angiogenesis regulators, fetal bovine serum (FBS) and histone deacetylase (HDAC) inhibitor, trichostatin A (TSA), were used to study the gene expression changes in HT1080 cells (Kim *et al.*, 2001).

The validation of the functionality of Angio_Chip was performed through three steps, *i.e.* scanning image analysis, signature correlation analysis, and microarray data validation using gene expression profiling. In the first step of validation, the scanning image of the examined Angio_Chip was analyzed as shown in Figure 2A. Duplicate spot images of FBS signatures (FBS^{1st} and FBS^{2nd}) were well correlated with each other. Also in TSA signatures, each duplicate image was exactly same. Magnified spots are representative

Figure 1. Experimental schemes of cDNA chip analysis. Figures show HT1080 cells treated with indicated reagents and experimental conditions. (A) FBS signature represents cDNA chip data from control (cy3) versus FBS treatment (cy5). (B) TSA signature represents cDNA chip data from FBS treatment (cy3) versus FBS and TSA co-treatment (cy5).

signatures from each experimental condition, which show correlation between each duplicate signature and remarkable difference between two experimental conditions.

In the second step, signature correlation analysis was performed using both hierarchical clustering of gene expression profiles and signature correlation coefficient value (ρ). GeneSight data analysis software (v3.5) was used for hierarchical clustering. As shown in Figure 2B, each duplicate signature in two experimental conditions, FBS^{1st} vs FBS^{2nd} and TSA^{1st} vs TSA^{2nd} were well correlated from the clustering analysis. Signature correlation coefficient value (ρ) has been used for determining a correlation degree between two different gene expression profiles quantitatively (Marton *et al.*, 1998). Signature correlation coefficient values for FBS^{1st} vs FBS^{2nd}, TSA^{1st} vs TSA^{2nd}, and FBS^{1st} vs TSA^{1st} were 0.81, 0.76, and < 0.1 , respectively. High correlation values (magnitude of $\rho > 0.7 \pm 0.1$) are very significantly different from zero, and small correlation values (magnitude of $\rho < 0.2$) are considered as not significant. Each correlation coefficient value was also demonstrated by scatter plot analysis, as shown in Figure 2C. Highly correlated signatures were shown as spots forming a diagonal line.

In the third step of validation, gene expression data were analyzed and compared with previously reported data. Among 153 angiogenesis related genes, 7

Table 2. Gene expression profiles in response to TSA.

Up-regulated genes by TSA		Down-regulated genes by TSA	
Gene name	Ratio (Log ₂ cy5/cy3)	Gene name	Ratio (Log ₂ cy5/cy3)
plakoglobin	1.4	HDAC 7B	-2.0
STAT3	1.3	IL-1 β	-1.5
TSP-1	1.3	bFGF	-1.4
HDAC 3	1.2	Flt1	-1.1
p21, INK1A	1.1	UPAR	-0.8
INK3	1.1	VEGF C	-0.8
TSP-2	1.1	-	-

were selected as up-regulated genes and 6 were down-regulated genes in response to TSA (Table 2). The selected genes were further validated using RT-PCR analysis (Figure 3). Among the selected up-regulated genes, junction plakoglobin and p21^{WAF1} are well known HDAC target genes (Hassig *et al.*, 1997; Shim *et al.*, 2004). Thrombospondin 1 (TSP-1) has been reported recently as an HDAC target gene (Cinatl *et al.*, 2002). In the down-regulated genes, VEGFs are known to be decreased by HDAC inhibitors (Kim *et al.*, 2001; Deroanne *et al.*, 2002). In addition, Flt1 and UPAR were reported to be down regulated by a depsipeptide HDAC inhibitor, FK228

Figure 2. Microarray data validation. (A) Scanning image analysis of cDNA chip data. Duplicate microarray images from each experimental condition (FBS and TSA) are shown. *Left panel*; scanning image of FBS signature. Each duplicate signature is designated as FBS^{1st} and FBS^{2nd}. Magnified images are representative spots in each duplicate. *Right panel*; duplicate scanning images from TSA signature. (B) Hierarchical clustering of each duplicate signature in two experimental conditions is shown. (C) Scatter plots and signature correlation coefficient values (p) of each duplicate signature (*left and middle panels*). The *right panel* represents the signature correlation coefficient value (p) between two experimental conditions (FBS vs TSA).

Figure 3. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of TSA-responsive genes in HT1080 cells. Amplified RT-PCR fragments of up-regulated genes (*upper panel*) and down-regulated genes (*lower panel*) by TSA are shown. Actin RNA was used as an internal control.

(Kwon *et al.*, 2002). These results demonstrate that the gene expression profiles of several known HDAC target genes in Angio_Chip are well correlated with the previously reported data.

Discussion

The present study shows the development of the first angiogenesis-focused cDNA chip with duplicated spots. The genes arrayed on the Angio_Chip are fully listed on the web (<http://rmd.takara.co.kr>). Arrayed genes are all known genes involved in angiogenesis. Notably, angiogenesis-related genes which have been recently focused by many of scientists were selected. Conventional genome-wide DNA chips are very informative and provide a vast of gene expression information. However, this complicated genomic information often turn to be useless, if they are not analyzed adequately for specific research purposes. Alternatively, some of phenotype or disease-focused DNA chips, such as Cancer gene-focused DNA chip, are very useful, because a small number of genes can provide the accurate and reproducible data (Kim *et al.*, 2002; Shim *et al.*, 2004). Moreover, Angio_Chip has duplicate spots on a slide which can provide more reliable data from DNA microarray analyses. From the validation experiments, all duplicate signatures in Angio_Chip are well correlated with each other demonstrating that the duplicate spots on the chip are very effective to generate reproducible data from DNA microarray analyses. We finally examined whether the microarray data are consistent with previously reported results. The data sets from TSA signature were used for this study, because HDAC is a well known direct transcriptional regulator and many of its target genes have been reported (Hoshikawa *et al.*, 1994; Grunstein, 1997). Some known HDAC target genes are also detected in the microarray data and the expression profiles of the genes are well correlated with previously reported data. Several other genes were also regulated in their gene expression by TSA but these have not been reported yet. Further studies on these new HDAC target genes would be important for

deciphering new roles of HDAC in angiogenesis.

In conclusion, the present study demonstrates that the Angio_Chip is a functional DNA chip, which will be very useful tool for angiogenesis-related basic and applied studies.

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