Detection of Mycoplasma Infection in Cultured Cells on the Basis of Molecular Profiling of Host Responses

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Abstract

Adaptive responses to diverse microbial pathogens might be limited in relatively few types. Host cell responses to pathogens are believed to be patterned or stereotyped along with species or class. We tried to compose the host response to Mycoplasma in terms of cellular gene expression. Although gene expression profile of two host HeLa and 293 cells were quite different each other, 30 genes were differentially expressed by mycoplasma infection in both of HeLa and 293 cells. Six of them (PR48, MADH4, MKPX, CRK, RBM7, NEK3) were related to cell cycle or proliferation. Another category of genes like IL1HY1, KLRF1, TNFSF14, GBP1 were host defense to elicit immune responses. With this set of genes, we establish the prediction model for mycoplasma contamination.

Keywords: Mycoplasma, oligonucleotide microarray, prediction model, biomarker

Introduction

The Mycoplasma, with over 100 different species, are the smallest self-replicating organisms known at present and constitute a distinct class within the prokaryotes characterized by their lack of a rigid cell wall. They can be classified into fermentative strains, which gain energy by fermentation of carbohydrates and non-fermentative strains that are unable to metabolize carbohydrates via glycolysis. The mycoplasmas are extra cellular parasites usually attached to the external surface of cells, but can also penetrate these (Razin *et al.*, 1998). In humans, *M. pneumoniae* is a frequent cause of respiratory infections, and is at the origin of approximately 20 % of all community-acquired pneumonias (Hammerschlag, 2001). Being 'minimal cells', Mycoplasma have also been used to investigate the machinery of self-replicating organisms (Razin, 1992).

Beside health problems, mycoplasma contamination constitutes one frequent problem when studying cultured cells (estimated frequency varying from 5 to 35 %). The strains *M. hyorhinis, M. orale, M. arginini, M. fermetans, M. hominis* and *Acholeplasma laidlawii* represent 90-95 % of the contaminating isolates (Hay *et al.*, 1989). Contamination is initially often difficult to detect because the contaminated culture grows well and appears normal by ordinary light microscopy. In addition, Mycoplasma is highly contagious and can rapidly spread through the cell stocks. The possible consequences of mycoplasma infection for the host-cells are multiple and variable, ranging from no apparent effect to extensive changes with inhibition of cell proliferation, induction of apoptosis, induction of cytokines and oxidative radicals, and malignant transformation (Avron and Gallily, 1995; Sokolova *et al.*, 1998; Tsai *et al.*, 1995). There is also a possibility that mycoplasmal biological activities may erroneously be interpreted as being of host origin (Choi *et al.*, 1998).

Understanding the molecular basis of a host's response to microbial infection is essential for preventing disease and tissue damage as a result of the inflammatory response. A better understanding of this process should allow for the design of drugs that can more specifically and effectively target infected cells with reduced side-effects. The host-pathogen interaction can result in many changes to the host cell including modulation of RNA expression, target receptor induction, actin cytoskeletal rearrangements, signal transduction pathway activation, and vacuolar trafficking (Finlay and Cossart, 1997; Cummings and Relman, 2000). DNA microarray enabled us to describe a unique biological phenomenon in terms of genome-wide gene expression analysis. It can provide a bird-eye view on a certain phenomenon as well as complete list on the significant genes. Gene expression profiling using DNA microarray has been offering potentials for defining the patterns especially on gene expression during normal biological or disease processes. Moreover, many of differentially expressed genes that may play an integral role in these processes were identified.

In this paper, we have utilized spotted oligonucleotide microarray to examine the expression of 10,416 known

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Accepted 9 June 2005

regulatory genes upon mycoplasma infection. We compare the distribution of patterns of gene expressions in two different cell lines, HeLa and 293 cells. After that we select 30 genes that expressed differentially in both of HeLa and 293 cells, as biomarkers for Mycoplasma infection. Actual prediction model for the infection is also discussed.

Materials and Methods

Cell culture and Mycoplasma infection

We used HeLa and 293 cells were maintained in Dulbeccos modified Eagle medium (DMEM, JBI, Seoul, Korea) supplemented with 10 % fetal bovine serum (JBI), 1 % Penicillin-Streptomycin (Invitrogen). Cells were plated in concentration of 2~5×10^6 per T75 flask 18 h before mycoplasma infection. *M. hyorinis* were harvested at log phase to treat onto cells at 10^8 per T75 flask. After 48 h, cells were washed with phosphate buffered saline and harvested for RNA preparation.

RNA extraction

Total cellular RNA was prepared using RNAeasy total RNA purification kit (Qiagen, Valencia, CA) followed by treatment with RNase-free RQ DNase (Promega Corp., Madison, WI). For cDNA synthesis, random hexamer primers (Gibco/BRL) were used to prime reverse transcriptase (RT) reactions. Using this method it was possible to use the same RT reaction (cDNA) for PCR amplification with different sets of gene-specific primers. The cDNA synthesis was carried out using Moloney murine leukemia virus (M-MLV) Superscript II reverse transcriptase (Gibco/BRL) following the manufacturer's instructions. To analyze relative expression of different mRNAs, the amount of cDNA was normalized based on the signal from ubiquitously expressed actin mRNA.

Oligonucleotide microarray

Total RNAs from control or Mycoplasma-infected HeLa or 293 cells used as to extract total RNA for quantiplicated experiment including one dye-swapping set.

Total RNA was prepared by using TriZol reagent (Gibco-BRL). The array used in this experiment was MacArray (Oligo-Human 10K, Macrogen Inc., Seoul, Korea) according to the manual provided by manufacturer. Briefly, 100 μg of total RNA was labelled by incubating it with Cyanine-3-dUTP (6.0 mM) (Perkin Elmer Life Sciences) or Cyanine-5-dUTP (4.0 mM) (Perkin Elmer Life Sciences), dNTP mixture, 0.1M DTT, RnaseOUT, inorganic pyrophosphatase, reverse transcriptase at 40 °C for 3 hours. Before hybridization, 8 μg of Cyanine-3-CTP labelled cRNA and 8 μg of Cyanine-5-CTP labelled cRNA were mixed together with 2.5 μl of Mouse Cot-1 DNA (Invitrogen), 2.5 μl of Deposition control target (Operon Technologies), and 12.5 μl of 2Xhybridization buffer (Agilent Technologies). The mixture was centrifuged at 12,000 rpm for 3 min after it was boiled for 2 min. About 24 μl of the mixture was hybridized on the array at 65 °C for 16 h. After hybridization, the array was washed with solution I (0.5×SSC, 0.01% SDS, and 1 mM DTT in nuclease-free water), solution II (0.25×SSC, 0.01% SDS, and 1 mM DTT in nuclease-free water), and solution III (0.06×SSC and 1 mM DTT in nuclease-free water) respectively. Cy3 and Cy5 fluorescent intensities were determined using the GenePix scanner (Axon Instruments), and images were analyzed using the built-in program to calculate relative ratios and to determine confidence intervals.

Data analysis

Fluorescence intensity was processed and measured using GenePix Pro software (Axon Instruments). Intensity data were imported to an in-house microarray database. Variance stabilizing normalization by Huber *et al.* was applied with the 'vsn' package in Bioconductor using the R statistical package. After performing intensity-dependent global LOWESS regression, spatial and intensity dependent effects were managed by pin-group LOWESS normalization following by the approach of Yang *et al.* A variation filter was used to reduce the number of genes for the following cluster analysis. Specifically, we applied the non-parametric Kruskall-Wallis one-way analysis of variance to eliminate the genes that did not change significantly across samples.

Results

Characteristics of host response to Mycoplasma

Host responses to mycoplasma infection were tested in two different human cell lines, HeLa and 293, which is usually used by molecular biology experiments. Subconfluent cells at 2~5×10^6 cells per 100 mm dish were incubated with mycoplasma pellet at the concentration of 10^8 per 10 ml media. Gross morphology of each cells was not significantly changed upon microbial infections. To stabilize the host-pathogen interaction, we incubated cells with Mycoplasma for 48 h. The transcripts of infected cells were compared with uninfected control cells.

Overall expression similarities for each samples of microarray data is provided in Fig. 1. These measures in the table are calculated as a pattern-similarity of two samples. The absolute value of Pearson correlation coefficient is used for the pattern-similarity. According to

293-1	0.772	0.695	0.732	0.462	0.466	0.603	0.304
	293-2	0.467	0.672	0.506	0.261	0.455	0.435
		293-3	0.498	0.301	0.433	0.437	0.589
			293-swap	0.297	0.308	0.564	0.411
				HeLa-1	0.631	0.649	0.801
					HeLa-2	0.851	0.698
						HeLa-3	0.723
							HeLa-swap

Fig. 1. Pattern-similarity table for mycoplasma infection of host response

Table 1. List of Genes that significantly changed upon mycoplasma infection

Category	GenBank NO	Locus Name	Description	Hela Control (Mean±SD)	HeLa Mycoplasma (Mean±SD)	293 Control (Mean±SD)	293 Mycoplasma (Mean±SD)	Control (Mean±SD)	Mycoplasma (Mean±SD)
cell cycle and growth	NM_013239	PR48	Protein Phosphatase 2a 48 kda regulatory subunit	-0.323 ±0.296	-0.778 ±0.183	-0.626 ±0.154	-0.754 ±0.173	-0.475 ±0.269	-0.766 ±0.165
	NM_005359	MADH4	mad, mothers against decapentaplegic	-0.343 ±0.130	-0.749 ±0.305	-0.501 ±0.221	-0.636 ±0.196	-0.422 ±0.184	-0.693 ±0.245
			homolog 4(drosophila)	0.058 ±0.411	-0.854 ±0.607	-0.309 ±0.267	-0.695 ±0.498	-0.125 ±0.369	-0.775 ±0.521
	NM_020185	MKPX	mitogen-activated protein kinase phosphatase x	-0.437 ±0.361	-0.811 ±0.359	-0.592 ±0.336	-0.771 ±0.073	-0.514 ±0.323	-0.791 ±0.241
	NM_016823	CRK	v-crk avian sarcoma virus ct10 oncogene homolog isoform a	-0.050 ±0.069	-0.796 ±0.318	-0.141 ±0.088	-0.620 ±0.206	-0.096 ±0.087	-0.708 ±0.265
	NM_016090	PBM7	rna binding motif protein 7						
	Z29067	NEK3	protein kinase	0.059 ±0.109	-0.585 ±0.401	-0.167 ±0.156	-0.771 ±0.230	-0.054 ±0.173	-0.678 ±0.319
immune response	NM_012275	IL1HY1	*interleukin-1 receptor antagonist homolog* 1	0.542 ±0.457	-1.113 ±0.458	-0.017 ±0.327	-0.663 ±0.444	0.263 ±0.469	-0.888 ±0.482
	NM_016523	KLRF1	killer cell lectin-like receptor f1	0.123 ±0.259	-0.792 ±0.197	0.015 ±0.293	-0.951 ±0.629	0.069 ±0.254	-0.871 ±0.440
	NM_003807	TNFSF14	tumor necrosis factor(ligand) superfamily, member 14	-0.521 ±0.276	-0.637 ±0.328	-0.619 ±0.456	-0.631 ±0.309	-0.570 ±0.342	-0.634 ±0.295
	NM_002053	GBP1	guanylate binding protein 1, interferon-inducible, 67kd	-0.146 ±0.082	-0.547 ±0.432	-0.573 ±0.422	-1.063 ±0.275	-0.360 ±0.358	-0.805 ±0.434
metabolism	NM_013410	AK3	adenylate kinase 3	0.382 ±0.286	-0.964 ±0.428	-0.169 ±0.356	--0.398 ±0.125	0.107 ±0.147	-0.681 ±0.420
	NM_005328	HAS2	hyaluronan synthase 2	0.022 ±0.462	-1.132 ±0.365	0.112 ±0.233	-0.479 ±0.235	0.067 ±0.331	-0.806 ±0.450
RNA processing	NM_015969	MRPS1	mitochondrial ribosomal protein s17	-0.310 ±0.246	-0.625 ±0.197	-0.298 ±0.279	-0.652 ±0.126	-0.304 ±0.235	-0.639 ±0.154
	NM_003134	SRP14	signal recognition paticle 14kd(homologous alu rna binding protenin)	-0.376 ±0.309	-0.082 ±0.379	-0.518 ±0.190	-0.699 ±0.030	-0.477 ±0.242	-0.759 ±0.257
	NM_018702	ADAR3	double-stranded rna specific adenosine deaminase	0.470 ±0.183	-0.784 ±0.313	-0.169 ±0.444	-0.766 ±0.412	0.150 ±0.464	-0.775 ±0.339
	NM_022052	NXF3	nuclear rna export factor 3	-0.722 ±0.147	-0.582 ±0.559	-0.396 ±0.207	-0.787 ±0.212	-0.559 ±0.240	-0.684 ±0.407
signaling	NM_005912	MO4R	melanokortin 4 receptor	-0.306 ±0.410	-0.987 ±0.065	-0.610 ±0.123	-0.656 ±0.299	-0.458 ±0.318	-0.821 ±0.267
structure	NM_002055	GFAP	glial fibrillary acidic protein	-0.182 ±0.092	-0.851 ±0.400	-0.303 ±0.217	-0.606 ±0.075	-0.243 ±0.163	0.728 ±0.297
	NM_022141	PARVG	gamma-parvin	-0.154 ±0.089	-1.008 ±0.329	0.157 ±0.422	-0.557 ±0.190	0.001 ±0.322	-0.783 ±0.346
transport	NM_025243	SLC19A3	solute carrier family 19, member 3	0.430 ±0.397	-0.785 ±0.290	0.217 ±0.265	-0.629 ±0.485	0.323 ±0.324	-0.707 ±0.379
	NM_000452	SLC10A2	solute carrier family 10 (sodium/bile acid cotransporter family), member 2	0.010 ±0.164	-0.888 ±0.619	0.089 ±0.289	-0.528 ±0.313	0.049 ±0.214	-0.708 ±0.493
	NM_004731	SLC16A7	solute carrier family 16 (monocarboxylic acid transporters), member 7	0.654 ±0.357	-0.801 ±0.426	-0.228 ±0.500	-0.492 ±0.213	0.213 ±0.620	-0.646 ±0.353
	BC004275		prosaposin (variant gaucher disease and variant metachromatic leukodystrophy)	-0.186 ±0.044	-0.773 ±0.449	-0.249 ±0.509	-0.620 ±0.472	-0.217 ±0.325	-0.697 ±0.435
unknown	XM_071646	LOC139674	hypothetical protein xp_071646	-0.195 ±0.194	-0.663 ±0.221	-0.323 ±0.089	-0.802 ±0.446	-0.259 ±0.152	-0.732 ±0.335
	NM_014868	RNF10	ring finger protein 10	0.518 ±0.604	-1.179 ±0.250	0.097 ±0.144	-0.701 ±0.213	0.307 ±0.455	-0.940 ±0.334
	NM_004907	ETR101	immediate early protein	-0.370 ±0.198	-0.854 ±0.463	-0.263 ±0.087	-0.810 ±0.373	-0.316 ±0.149	-0.832 ±0.390
	NM_022804	SNURF	snrpn upstream reading frame protein	0.073 ±0.088	-0.899 ±0.391	-0.229 ±0.279	-0.754 ±0.282	-0.078 ±0.248	-0.826 ±0.325
	NM_007021	DEPP	decidual protein induced by progesterone	-0.556 ±0.003	-0.925 ±0.580	-0.600 ±0.076	-0.711 ±0.581	-0.578 ±0.054	-0.818 ±0.550
	ENSG00000065883		ensembl prediction	-0.285 ±0.131	-0.679 ±0.364	-0.361 ±0.057	-0.607 ±0.316	-0.323 ±0.099	-0.643 ±0.318
	AF077047		galanin-related peptide	-0.339 ±0.224	-0.739 ±0.237	-0.761 ±0.317	-0.579 ±0.273	-0.550 ±0.337	-0.659 ±0.252

the figure, we observe that characteristics of host response to mycoplasma have remarkable difference in two different cell lines.

Selection of biomarkers for Mycoplasma infection

From the quadruplicated profile of 10K oligonucleotide microarray in each cells, we selected genes for the host responses to mycoplasma infection under the following standards (Table 1) :

i) Standard deviation in both of infected and control groups should be less than two fold in both cell lines, ii) Average fold activation of infected groups should exceed two fold. iii) Average fold activation of uninfected control group should be less than two fold. iv) Activation of gene

Table 2. Contamination indices for each samples

cell - replication	Contamination indices for 30 significantly selected genes	Contamination indices for randomly selected genes (Mean ± SD)	Thresholds (Mean+3*SD)
293 cell-1	0.35248	0.1179 ± 0.0528	0.2763
293 cell-2	0.16773	0.0857 ± 0.0431	0.2150
293 cell-3	0.19126	0.0695 ± 0.0383	0.1844
293 cell-swap	0.11265	0.0191 ± 0.0197	0.0782
HeLa cell-1	0.20052	0.0711 ± 0.0411	0.1944
HeLa cell-2	0.21936	0.0518 ± 0.0348	0.1562
HeLa cell-3	0.24699	0.1032 ± 0.0465	0.2427
HeLa cell-swap	0.17990	0.0533 ± 0.0347	0.1574
Average	0.20886 ± 0.0655	0.0714 ± 0.0495	0.2199

expression should be found in both of HeLa and 293 cells.

According to the GO (http://www.geneontology.org/), we could classify these 30 genes in their biological functions (Table 1). In view of biological effect of Mycoplasma infection, we found that two categories of genes like cell cycle/proliferation and immune response were changed upon the infection. Especially Mycoplasma leads to the growth retardation or arrest in vitro, which might be explained by the transcriptional activation of genes like PR48, MADH4 and MKPX. Although two cell lines were not from immunological tissues, we detected the increase of transcripts like IL1HY1, KLRF1, TNFSF14 and GBP1.

Prediction model for Mycoplasma contamination

The 30 genes selected significantly, listed in Table 1, are believed that they are strongly involved the process of mycoplasma infection in human cells. From this fact, we define the contamination index for a given sample of microarray, as follows:

$$CI(S) = \frac{1}{30} \sum_{i=1}^{30}$$

where {g1, ..., g30} is the set of gene in Table 1, M(g,s) is the log-ratio of expression of g under the sample s, and $f(x) = \frac{\exp(-5+5\,|\,x\,|)}{1+\exp(-5+5\,|\,x\,|}$ is the function t hat indicate the probability of a gene with expression value x became a differentially expressed gene.

Since these genes are differentially expressed in both cells, we may consider that the contamination index CI(s) reflects that whether the unknown sample s is infected by Mycoplasma or not. To give a specific criterion for mycoplasma contamination, we calculated the contamination indices with respect to the randomly selected genes. Mean values and standard deviations of the indices for each samples are extracted by iterating these process 1,000 times. Parameters in random model are listed in Table 2.

We set a criterion to predict contamination by Mycoplasma for a given sample s as follows:

$$CI(s) > m + 3^{*}\sigma,$$

where m and σ are mean and standard deviation in random model, respectively. The threshold "$m+3^{*}\sigma$" is taken to satisfy that the false positive rate is less than 0.01. That is, the probability of CI(s) > $m+3^{*}\sigma$ when s is not contaminated by Mycoplasma is less than 0.01. According to the Table 2, only the case of sample "293 cell-replication 2" fails to predict the mycoplasma contamination out of 8 total samples. In the sample "293 cell-replication 2", we observe that the contamination index (0.16773) is much larger than the mean contamination index (0.0857) for randomly selected genes, but the standard deviation (0.0431) is relatively large to the contamination index, so the log-expression profile in the sample is far from the normal distribution, which is a basic assumption of our predicting model.

Discussion

Efforts in functional genomics related to cancer research have yielded major successes in the pursuit of gene expression signatures. Approaches for gene expression analysis, such as time-series analysis, pattern discovery, clustering, and class prediction, have been recently reviewed (Solnim, 2002). Expression-based criteria or class predictors have been defined based on neighborhood analysis (Golub, 1999), a supervised method based on a subset of genes whose expression strongly correlates with specific classes, as well as Bayesian regression models (West, 2001) and artificial neural networks (Khan, 2001). These predictors were successfully used to classify novel samples in a manner consistent with clinical assessments. In fact, classifications based on gene expression alone or class discovery have also been

demonstrated and suggesting that gene expression profiling have the capacity to identify subtypes that have not been previously defined (Golub, 1999). Although these results are promising, one should note that many of the previously conducted cancer line gene expression analyses are one dimensional; in contrast, a host expression profile evoked by pathogen exposure would be expected to be temporal and may also exhibit dose dependence. Comprehensive sets of gene expression profiles that explore temporal and dose ranges for pathogen exposure must be produced to map the continuum of gene expression changes.

In this paper, we suggested a list of genes that is believed to strongly related to the mycoplasma contamination in two different human cell lines, HeLa and 293. We also suggested a model to predict that a given sample is infected by the mycoplasma. This naive process has strong power to predict, and is suitable to the our data which has unique structure that consisted of expression of genes upon infection in various experimental conditions such as cell-lines or dose ranges. To get a more strong prediction power for overall-non-contamination of a concerned sample, we need a structural experimental profiling data for host response of infection by wide source of pathogens.

Acknowledgment

This work was supported by a grant from Korea Food and Drug Administration.(KFDA-03092-LIF-010 and KFDA-04092-LIF-009)

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