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A vector method for comparison of RNA and protein expression levels of cytokines upon CD40L treatment of microglia

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Abstract

Understanding complex relationship between gene expression and its corresponding protein levels over time is critical for system level studies of biological processes. We developed a vector based method to understand the correlation between mRNA expression and its corresponding protein levels of different cytokines when microglia is treated with CD40L over different time points (1 hr and 6 hrs). Vectors are constructed using RNA probe intensity from Affymetrix GeneChip array and protein levels using multiplex bead based method (Bio-Plex, Biorad). The angles between protein and RNA vectors that represent different cytokines upon CD40L treatment of microglia were measured using vector dot products. We observed that a high number of cytokines have similar trend for both RNA and protein changes over time under treatment conditions.

Keywords: Correlation, Vector dot product, RNA, Protein, GeneChip, BioPlex

INTRODUCTION

Understanding the relationship between mRNA and corresponding protein expression levels is complex but essential to study molecular processes at system levels. The holy grail of biology is DNA transcribed to mRNA which is translated into a peptide. The peptide may undergo further post-translational modification and transport to form a functional protein. Each of these steps is controlled either directly or indirectly by positive or negative feedback signals (Liu *et al.*, 2004). Modern biology is concerned with delineating such signals and attempts to understand biological systems as a network of macromolecules that act in a quantitative fashion to achieve specific task (Cox *et al.*, 2007). Proteomics is the study of entire or fractional number of proteins, genetic complement of gene expression, and their quantitative or qualitative changes over time (Elrick *et al.*, 2006). Genomics on the other hand, deals with understanding large-scale mRNA levels and controls associated with gene expression (Ait-Ghezala *et al.*, 2005; Paris *et al.*, 2005; Crawford *et al.*, 2007). A direct correlation of mRNA measured with technologies like microarray to the protein levels measured using 2D-gel is rarely seen owing to a large amount of noise and difficulty in designing experiments taking into consideration the periodic sampling over large time points with several replicates (Nie *et al.*, 2007). There are no quick and easy way to correlate such expression patterns, although it is highly desirable to further perform hypothesis based study designs (Cox *et al.*, 2005; Fagan *et al.*, 2007).

A directional vector based on the quantitative estimation from genomics and proteomics experiments can be used to compare expression levels. Here we describe our approach using directional vectors for both mRNA and proteins for a limited set of genes and attempt to identify correlation between their expression levels for

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cytokines (Ait-Ghezala *et al.*, 2005). In this method we define directional vectors over time for RNA and Protein levels as measured using GeneChip (Affymetrix, CA) and Bioplex (Biorad, CA) technologies respectively for selected cytokines pre- and post-treatment. Then we use scalar product to measure the angle between them. Microglial cells are activated when they are treated with CD40L and produce a set of cytokines responsible for inflammatory responses. Complex relationship between mRNA levels and protein levels of induced cytokines in activated microglia is largely unknown. Here we attempt to identify those cytokines for which mRNA and protein levels are correlated upon activation of microglia by CD40L.

MATERIALS AND METHODS

RNA preparations

Confluent human primary microglia cells (at passage 3 in 75 cm^2 flasks) were treated for 30 min in 10 ml of prewarmed microglia medium supplemented with 5% FBS and containing 2 μg/ml CD40L or the medium alone. Three 75 cm^2 flasks of confluent microglia cells were used for each condition. Following 30 min of incubation at 37 -C in 5% CO_2, RNA was extracted. Microglia cells from each 75 cm^2 flask were washed with PBS and trypsinized in 2 ml trypsin (Gibco-Invitrogen, CA). Cells were centrifuged and re-suspended in 4 ml lysis buffer RLT supplemented with 1 μl/ml of h-mercaptoethanol (Gibco) and purified using the RNeasy Midi kit (Qiagen, CA) according to the recommendations of the manufacturer. Quality control steps ensured that the RNA used for microarray and real-time RT-PCR analysis was of high quality and the quality and purity of the RNA obtained were tested on Agarose gels and spectrophotometrically at 260 nm and 280 nm. All RNA samples had an A260/280 absorbance ratio between 1.9 and 2.1. Preparation of cRNA, hybridization and scanning of probe arrays were performed according to the protocols of the manufacturer (Affymetrix, CA).

Oligonucleotide microarray analysis

The Affymetrix U133A microarray was hybridized and washed in the fluidic station using manufacturer specified (EukGE_WS2V4_450) wash protocol. The hybridized oligonucleotide chips were scanned using the GeneChip Scanner 3000 (Affymetrix, CA). The number of scan parameters was set to one and the pixel size was set to 2.5 AM. Raw images after the scan were visualized and inspected for image artifacts or improper grid alignment. A total of 6 Affymetrix U133A chips were used for this study: 3 for the controls and 3 for the CD40L treatment (30 min) of microglia. Low-level analysis to assign expression index was carried out using the MAS5.1 Microarray Suite (Affymetrix, CA). Global scaling of the measured intensities was performed by setting the average signal to a target signal value of 500. The statistical threshold a1 was set to 0.05 and a2 to 0.065 for the detection P value and MAS default detection algorithm was applied. This algorithm uses a discrimination score for each probe pair and tests the discrimination against a threshold s (0.015). The detection P value was calculated using a one-sided Wilcoxon's Rank test. If the detection P value is less than α1, a detection call of ''present'' was assigned and if it was greater than α2, then expression for that particular probe was considered to be ''absent''. We considered ''marginal'' assignment (detection p-values in between α1 and α2) also as ''present''. All the chips for this experiment were fault-free and had acceptable background values. None of the chips had a noise value (raw Q) greater than 5. All the spike-in hybrid controls (*bioB*, *bioC*, *bioD* and *cre*) were present in increasing intensities with comparable values across all chips. Each probeset scaled intensity values were calculated. The intensity index reflects quantity of RNA present across each sample.

Multiplex quantitative cytokine measurement

We investigated the effect of CD40 ligation on the production of cytokines and hemokines by primary cultures of human microglia using the Bio-Plex multiplexcytokine assay kit from Bio-Rad Laboratories, which combines the principle of a sandwich immunoassay with the Luminex fluorescent-bead-based technology allowing the simultaneous detection of multiple cytokines and chemokines in the same sample. Human primary microglia from the same batch used for the genomic study were treated with the same dose of 2 μg/ml of CD40L then incubated at 37 -C and media from these plates were collected after 1 and 6 h of treatment and tested for cytokine

and chemokine profiling using human cytokine 17-plex panel kits (Bio-Rad, CA).

RNA and protein level trend analysis for cytokines

In order to verify whether an increasing trend in RNA levels (R) between control (g=ctrl) and CD40L treatment of microglia (g=treat) over time correspond with protein levels (P), we constructed vectors using the data from 30 minutes (t=0.3 and t=1 hr for protein) and 6 hrs (t=6). We used the calculated intensity values of probe ids, corresponding to cytokines, from MAS5.1 as an indicator of RNA level. Protein levels (P) of different cytokines were measured using BioPlex. Using the intensities of microchip, vector $\vec{R}$ was constructed from the data points ($R_{g=ctrl,t=0.3}$, $R_{g=treat,t=0.3}$) and ($R_{g=ctrl,t=6}$, $R_{g=treat,t=6}$). 'R' represents GeneChip intensity for a particular cytokine, '***g***' is the group and '***t***' is time. This vector indicates the RNA trend over time upon treatment of microglia with CD40L. Similarly another vector $\vec{P}$ was constructed using the protein concentration (pg/mg) of different cytokines from BioPlex as data points: ($P_{g=ctrl,t=1}$, $P_{g=treat,t=1}$) and ($P_{g=ctrl,t=6}$, $P_{g=treat,t=6}$), where P is the protein concentration of a particular cytokine, '***g***' is group and '***t***' is time. The angle between the vectors $\vec{P}$ and $\vec{R}$ was calculated as follows:

$$\theta = \cos^{-1}\left(\frac{\vec{P} \bullet \vec{R}}{|\vec{P}||\vec{R}|}\right) \qquad \text{eq.1}$$

Where $\vec{P}$ and $\vec{R}$ are given by

$$\vec{P} = (P_{g=ctrl,t=6} - P_{g=ctrl,t=1})\,\hat{i} + (P_{g=treat,t=6} - P_{g=treat,t=1})\,\hat{j} \qquad \text{eq.2}$$

$$\vec{R} = (R_{g=ctrl,t=6} - R_{g=ctrl,t=.3})\,\hat{i} + (R_{g=treat,t=6} - R_{g=treat,t=.3})\,\hat{j} \qquad \text{eq.3}$$

and their corresponding unit vectors for representation is given by

$$\hat{P} = (P_{g=ctrl,t=6} - P_{g=ctrl,t=1})\frac{\hat{i}}{|\vec{P}|} + (P_{g=treat,t=6} - P_{g=treat,t=1})\frac{\hat{j}}{|\vec{P}|} \qquad \text{eq.4}$$

$$\hat{R} = (R_{g=ctrl,t=6} - R_{g=ctrl,t=.3})\frac{\hat{i}}{|\vec{R}|} + (R_{g=treat,t=6} - R_{g=treat,t=.3})\frac{\hat{i}}{|\vec{R}|} \qquad \text{eq.5}$$

Upon CD40L treatment of microglia, up-regulation of RNA expression for any cytokine will result in increasing trend that can be captured by vector direction. An increase in protein levels corresponding to the RNA trend can be confirmed if the angle between the vectors $\vec{P}$ and $\vec{R}$ is low. Thus one can correlate the trend of RNA expression with the protein levels using the angle between the vectors. Since RNA expression is quantified by intensity index and protein using concentration, for visualization purpose we show trend correlation for selected cytokine using normalized vectors (that has magnitude unity but has same direction as the parent vector, calculated using eq. 4, 5). The values for starting time point for both RNA and protein was scaled to zero.

RESULTS

The angles between protein and RNA vectors that represent different cytokines upon CD40L treatment of microglia were measured using equation 1. If the angle between the RNA and protein vectors was greater than 90°, we considered RNA and protein trend to lack correlation upon treatment of microglia with CD40L. Out of 17 cytokines measured using BioPlex, 12 of them had vector angles less than 90°. Using a proportion test, the observed ratio of 12/17 is different (p-value =0.146) from a random correlation of RNA and protein in which roughly half the cytokine members can be expected to correlate. The assumption of angle between the vectors to be less than 90° in order to infer correlation between RNA and protein levels is questionable if the vectors fall in different quadrants of the graph. Further, many of the cytokines measured do not change at RNA or protein levels, although the intensity index were provided in the Affymetrix chips

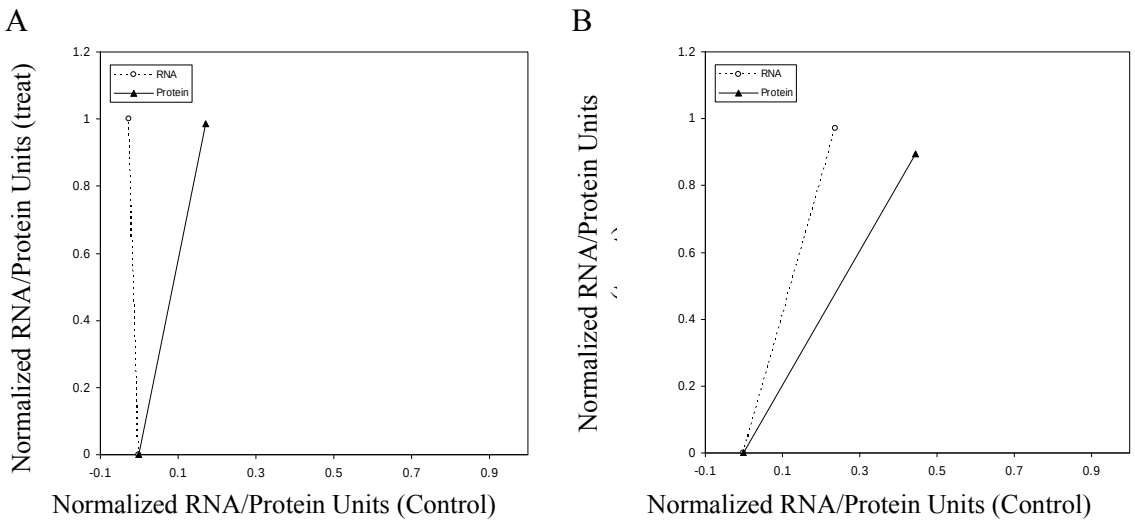

Figure 1: Plot of RNA and protein vectors of IL8 (A) and IL1b (B). X-axis is the normalized unit (eq 4, 5) that is dimensionle describing the RNA and protein levels of the control sample and the Y-axis is for the CD40L treatment. The data for the lowest ti point (30 min in case of RNA and 1 hr in the case of protein) is adjusted to be zero. If the protein and the RNA vector correlate w over time then one observes a close angle of separation between them. For IL8 (AffyID: 202859_x_at) the angle of separation is 1 and for IL 1b (AffyID:205067_at) 13°.

corresponding to cytokines: CSF2, CSF3, IL2, IL4, IL5, IL10, IL13, IL17 and TNF. The detection call associated with these cytokines was "absent" across all chips. This may be due to a very low or no expression of RNA and the stringent statistical cutoff set in MAS. Hence, in some situation a straight-forward correlation will not be possible between RNA and protein levels using our method; nevertheless it is helpful in identifying the similarity in RNA and protein trends. The angle between protein and RNA vector for IL8 and IL1b was less than 15° showing a strong correlation (Fig.-1).

DISCUSSION

Using protein levels for cytokines measured with BioPlex and the RNA levels measured with the microchip, we observed that a high number of cytokines have a similar trend for both RNA and protein changes over time under treatment conditions. IL8 increases from 2.3 fold RNA level at 30 minutes to 17.3 fold at 6hr when microglia was treated with CD40L. Corresponding trend in protein levels of IL8 is reflected by an increase from 2.23 fold at 1 hr to 5.5 fold at 6 hrs. In RNA levels, there was a decrease in the average intensity observed for control group at 30 min compared to 6 hrs. This is reflected in the protein vector (Fig.-1) in which the increase in the Y-axis (treatment) is steeper than X-axis (control). Our study establishes a relationship for protein and RNA levels of cytokine such as IL8 that is highly regulated by CD40L treatment of microglia. Our method intends to capture a corresponding increase (or decrease) in protein with RNA expression over selected time period. It is reasonable to use slight time point differences between RNA and protein expression levels as long as there is no significant change in the slope. Minor oscillations in expression levels over short period of time may be difficult to capture experimentally and more so to model it theoretically. A sufficient time (depending on the model system) should be allowed between end points during which trends can be analyzed using our method. We are developing new software tool that can capture maxima and minima of expression level to identify suitable cutoff points using discrete data collected over different time intervals. In general, modeling kinetics of RNA and Protein expression levels are complicated that will require several data points over a large time period. Although our method is not robust and has limitations it will still provide a reliable and quick way of answering correlation in genomic and proteomic expression levels that are regulated due to treatment conditions.

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