An Enrichment Method for Obtaining Biologically Significant Genes from Statistically Significant Differentially Expressed Genes in Comparative Transcriptomics

Panpaki Seekaki\textsuperscript{1} and Norichika Ogata\textsuperscript{1,2}

\textsuperscript{1} Nihon BioData Corporation, 3-2-1 Sakado, Takatsu-ku, Kawasaki, Kanagawa, Japan
\textsuperscript{2} Medical Mechanica Incorporated, 3-2-1 Sakado, Takatsu-ku, Kawasaki, Kanagawa, Japan
norichik@nbiodata.com

Abstract. Cells coordinate adjustments in genome expression to accommodate changes in their environment. A drug in culture media for \textit{in vitro} preclinical testing sometimes cause drastic regime shifting of genome expression system depending on the concentrations; e.g. primary cultured cells exposed to high concentrations of phenobarbital (≥1.0 mM) recovered their tissue-specific character as part of an individual organism. Drastic changes of transcriptomes interrupt discovering biologically significant genes in comparative transcriptomics. Here, we compared the amount of environmental changes and the amount of transcriptome changes using phenobarbital and the Chinese hamster ovary derived established continuous cell line CHO-K1; immortalized cell lines are accepted for \textit{in vitro} preclinical testing then primary cultured cells.

Keywords. Kolmogorov Complexity, Transcriptome, Information entropy.

1 Introduction

Gene screenings are performed in comparative transcriptomics using RNA-seq. Statistically significant differentially expressed genes were obtained from every comparison between any transcriptomes. Some p values from a comparison of one replicate with another one must be below 0.05 in comparative transcriptomics with Type-I error control. Between replicates, no genes are truly differentially expressed, and the distribution of p-values is expected to be uniform in the interval [0,1] (Anders & Huber 2010). We had reviewed and accepted papers performing differentially gene expression analyses between samples, listing over hundreds of statistically significant differentially expressed genes and introducing studies dealing with functions of those genes. Someone performed meaningless Gene Ontology analyses or pathway analyses except a few studies (Goecks et al. 2013). A few studies validated listed statistically significant differentially expressed genes (Lee et al. 2016), since most biologists may think there are few biologically significant genes in the statistically significant differentially expressed genes in comparative transcriptomics.
The problems of the comparisons in biology were discussed for a long time (Goethe 1820). Goethe pointed the problem of the comparisons in Osteology, osteologists may for the present state maintain that the division of the human bony structure was merely accidental; so that in descriptions, sooner or later, fewer bones were assumed, each describing them as they pleased and their own order. Finally, one wanted to deny man his “intermediate bones”. He remarks that his osteological studies awakened in him the desire “to establish a type according to which all mammals would be able to be examined for their similarities and differences.” (Gray 2004). We have to discover that for a meaning-full comparison, which are common to all subjects, and in which these parts are different. If we compared a human hand bone and a monkey's head bone, we would obtain many statistically significant differences and few biological significant differences. We have to compare the same bones of human and monkey and it is clear in Osteology, but not in comparative transcriptomics. A type of transcriptomes does not exist.

Previous studies indicated that the genome expression system displays a drastic regime shift (Luscombe et al. 2004; Noori 2014). Distinguish common and different is difficult in comparative transcriptomics. However, it was possible to judge transcriptomes into same regime or under different regime. Comparative transcriptomics for in vitro preclinical testing are widely performed as an alternative to animal tests. Determination of the drug concentration is critically important for in vitro drug-exposure testing in preclinical toxicology. High drug concentrations can induce a radical transcriptome response, while mild concentrations can make it difficult to determine cell responses. Ideally, a drug concentration should be high enough to induce the desired main effect, while not eliciting too many side effects. Previously, we had been forced to determine appropriate drug concentrations by considering cell morphology and through various other examinations.

A previous study established a quantitative method to determine the drug concentrations that should be used for in vitro preclinical testing using Shannon’s information entropy of transcriptome data, judging transcriptomes into same regime or under different regime (Ogata et al. 2015). Shannon’s information entropy a dimensionality reduction method was applied to obtain index of regime of the transcriptome. Dimensionality Reduction methods e.g. Principle component analysis and t-SNE had been used to transcriptome analyses, but biological meanings of value of results of these methods was unclear. Shannon’s information entropy also reduces the dimensions of transcriptome data and the biological meaning is clear; a cellular dedifferentiation increases Shannon’s information entropy of transcriptome data of the cells and the cellular differentiation decreases them (Ogata et al. 2012). The previous study discussed the relationship between the amount of transcriptome change and the amount of environmental change using silkworm fat body tissues cultured with several concentrations of phenobarbital and primary cultured cells exposed to high concentrations of drug recovered their tissue-specific character as part of an individual organism.

Previously, a perfect drug concentration was determined for primary cultured tissues. However, an industrial applicability of Shannon’s information entropy is questionable, since established cell lines mainly accepted for in vitro preclinical testing.
Established cell lines partially lose their tissue-specific character as part of an individual organism; for example, transplanted HEK293T cell line derived pluripotent cells formed tumor-like structures contained ectoderm and mesoderm tissues in dorsal flanks of kidney in busulfan-treated mice, however, no typical endoderm structures (Lin et al. 2008; Wang et al. 2014). A follow-up study on regime shift using immortalized cell lines is needed.

Even from a theoretical point of view, there is room for argument the application of Shannon’s information entropy on transcriptome analyses, since Shannon’s information entropy ignore zero count data contained transcriptome data obtained using RNA-seq. Recent study estimated Kolmogorov complexity of transcriptome treating zero count data (Seekaki & Ogata 2017). The elementary theories of Shannon information and Kolmogorov complexity have a common purpose (Grunwald & Vitanyi 2010). Kolmogorov complexity is the minimum number of bits from which a particular message or file can effectively be reconstructed. Since zero count data contained transcriptome data may have some message, we should treat zero count data using Kolmogorov complexity. Similar transcriptomes that were not distinguished using Shannon’s information entropy were distinguished using Kolmogorov complexity.

Here, we performed a comparison between the amount of transcriptome change and the amount of environmental change using an immortalized cell line CHO-K1 derived from Chines Hamster Ovary cells cultured with several concentrations of phenobarbital. We calculated Shannon’s information entropy and Kolmogorov complexity of transcriptome data. We also obtained phenobarbital-induced genes from a differentially expressed gene analysis.
2 Materials and Methods

All of the chemicals used in this study were of analytical grade. Phenobarbital sodium (Wako Pure Chemical, Osaka, Japan) was dissolved in distilled water to make a stock solution, which was added to the medium to make final concentrations of 0.25 and 2.5 mM phenobarbital. The original medium was replaced with phenobarbital-containing medium in the induction assays. CHO-K1 cells obtained from ATCC were incubated with 0, 0.25 and 2.5 mM phenobarbital for 10 hours. Induction assays were terminated by using QIAshredder (Qiagen, Basel, Switzerland), and the tissues were kept at –80°C until analysis. RNA was extracted using a commercial kit (RNeasy mini kit; Qiagen, Basel, Switzerland). We prepared a library for conventional RNA-seq using a commercial kit (TruSeq RNA Sample Kit; Illumina, San Diego, CA, USA) in accordance with the manufacturer’s protocol. We sequenced libraries for conventional RNA-seq using a commercial sequencer (HiSeq 2000; Illumina) in accordance with the manufacturer’s protocol. Short-read data have been deposited in the DNA Data Bank of Japan (DDBJ)’s Short Read Archive under project ID DRA005765. All raw sequencing reads were mapped to CHO-K1 RefSeq assembly (ID GCF_000223135.1) using bowtie (version 0.12.8) with the modified parameters (-l 75 -n 2 -p 4). Shannon’s entropy of transcriptome data and Kolmogorov complexity were measured as previously described (Ogata et al. 2015; Seekaki & Ogata 2017). All of the data were processed using bash (version 3.2) and visualized using R. Linear regression analyses were performed using R and Fitting Linear Models (lm). Differentially expressed gene (DEG) analyses were performed using R and the TCC package (version 1.1.99) (Sun et al. 2013).

3 Results and Discussion

To investigate the effects of phenobarbital concentration on Shannon’s information entropy and Kolmogorov complexity of transcriptome data, we sequenced 9 transcriptomes from CHO-K1 cells exposed to phenobarbital. Freshly subcultured cells (2×10⁶) were cultured for 24 hours in DMEM/F12 medium, and then cultured for 14 hours in medium supplemented with 0, 0.25 and 2.5 mM phenobarbital. We measured the Shannon’s information entropy and Kolmogorov complexity of those transcriptomes. Shannon’s entropy of transcriptome data and Kolmogorov complexity were measured as previously described (Ogata et al. 2015; Seekaki & Ogata 2017). All of the data were processed using bash (version 3.2) and visualized using R. Linear regression analyses were performed using R and Fitting Linear Models (lm). Differentially expressed gene (DEG) analyses were performed using R and the TCC package (version 1.1.99) (Sun et al. 2013).
Kolmogorov complexity was better index of transcriptome data than Shannon’s information entropy. Kolmogorov complexity changed between phenobarbital concentrations of 0.25 and 2.5 mM (Figure 1b). In previous study, cells cultured in media containing lower drug concentrations than the tipping point showed uniformly high Shannon’s information entropy, while those cultured at higher drug concentrations than the tipping point showed uniformly low Shannon’s information entropy. However, in this study, cells cultured in media containing low drug concentrations showed low Shannon’s information entropy and those cultured at high drug concentrations showed low Shannon’s information entropy. This contradiction indicating that there is unknown mechanisms in a drastic regime shift of the genome expression system. Traditional RNA-seq measures means of cultured cells and the changes of cellular heterogeneity also changes the RNA-seq results. There is a possibility that phenobarbital induction make increase cellular heterogeneity of immortalized cell lines. Single cell transcriptome analyses of cells displaying a drastic regime shift would discover this problem.

Fig. 1. Comparisons between the amount of transcriptome change and the amount of environmental change. (a) Scatter plot of drug concentration vs Shannon’s information entropy. Transcriptomes of CHO-K1 cells that were cultured 10 hours in DMEM/F12 media supplemented with 0, 0.25 and 2.5 mM phenobarbital. (b) Scatter plot of drug concentration vs Kolmogorov complexity. Transcriptomes of CHO-K1 cells that were cultured 10 hours in DMEM/F12 media supplemented with 0, 0.25 and 2.5 mM phenobarbital.

We compared transcriptomes with close Kolmogorov complexity focusing on differentially expressed genes. In this study, a comparison between the transcriptome of a control group (0 mM phenobarbital) and that of an experimental group treated with the lowest phenobarbital concentration (0.25 mM) represented the comparison of close Kolmogorov complexity (Figure 2a). Indeed, only single differentially expressed gene was detected in the comparison between the control and the 0.25 mM phenobarbital-treated group. On the other hand, 305 genes were detected in comparisons between the control group and the 2.5 mM phenobarbital groups (Figure 2b).
The single differentially expressed genes detected in the comparison between the control and the 0.25 mM phenobarbital-treated group was *Cricetulus griseus* early growth response 1 (Egr1), transcript variant X1, mRNA. Early growth response 1 performs the essential roles for nuclear receptor CAR to activate the human cytochrome P450 2B6 gene (Inoue & Negishi 2008; Inoue & Negishi 2009). We could obtain the biologically significant gene from the comparison between the transcriptomes having close Kolmogorov complexity.

![Fig. 2. Differentially expressed gene analyses between transcriptomes.](image-url)

**Fig. 2. Differentially expressed gene analyses between transcriptomes.** (a) Scatter plot of differentially expressed gene analyses between Controls vs. 0.25 mM Phenobarbital experiment. (b) Scatter plot of differentially expressed gene analyses between Controls vs. 2.5 mM Phenobarbital experiment. (c) Scatter plot of differentially expressed gene analyses between 0.25 mM Phenobarbital experiment vs. 2.5 mM Phenobarbital experiment.

Previous study comparing between the amount of environmental change and the amount of transcriptome change corroborated plasticity of Shannon’s information entropy of transcriptome data and hysteretic phenomenon. The hysteretic phenomenon provides evidence of the bi-/multi-stable system. In this study, we did not examine hysteretic phenomenon although the plasticity of transcriptomes was indicated by cultivations of CHO-K1 cells in medium without phenobarbital after previous cultivation using medium containing 0.25 or 0.25 mM phenobarbital; Shannon’s Information entropy were 12.01561 (0.25mM->0mM) and 11.98386 (2.5mM->0mM); Kolmogorov complexity were 0.0640789 (0.25mM->0mM) and 0.0640927 (2.5mM->0mM). Further experiments were needed.
References

1. Anders S, and Huber W. 2010. Differential expression analysis for sequence count data. Genome biology 11:R106. 10.1186/gb-2010-11-10-r106
2. Goecks J, Mortimer NT, Mobley JA, Bowersock GJ, Taylor J, and Schlenke TA. 2013. Integrative approach reveals composition of endoparasitoid wasp venoms. PloS one 8:e64125. 10.1371/journal.pone.0064125
3. Goethe JW. 1820. Erster Entwurf einer allgemeinen Einleitung in die vergleichende Anatomie. Zur Morphologie I. http://anthrowiki.at/Bibliothek.Goethe/Naturwissenschaft/Erster_Entwurf_einer_allgemeine
4. Gray RT. 2004. About Face: German Physiognomic Thought from Lavater to Auschwitz. Wayne State University Press.
5. Grunwald P, and Vitanyi P. 2010. Shannon Information and Kolmogorov Complexity. http://homepages.cwi.nl/~paulv/papers/info.pdf
6. Inoue K, and Negishi M. 2008. Nuclear receptor CAR requires early growth response 1 to activate the human cytochrome P450 2B6 gene. J Biol Chem 283:10425-10432. 10.1074/jbc.M800729200
7. Inoue K, and Negishi M. 2009. Early growth response 1 loops the CYP2B6 promoter for synergistic activation by the distal and proximal nuclear receptors CAR and HNF4alpha. FEBS Lett 583:2126-2130. 10.1016/j.felslet.2009.05.031
8. Lee N, Shin J, Park JH, Lee GM, Cho S, and Cho BK. 2016. Targeted Gene Deletion Using DNA-Free RNA-Guided Cas9 Nuclease Accelerates Adaptation of CHO Cells to Suspension Culture. ACS Synth Biol 5:1211-1219. 10.1021/acssynbio.5b00249
9. Lin SL, Chang DC, Chang-Lin S, Lin CH, Wu DT, Chen DT, and Ying SY. 2008. Mir-302 reprograms human skin cancer cells into a pluripotent ES-cell-like state. RNA 14:2115-2124. 10.1261/rna.1162708
10. Luscombe NM, Babu MM, Yu H, Snyder M, Teichmann SA, and Gerstein M. 2004. Genomic analysis of regulatory network dynamics reveals large topological changes. Nature 431:308-312. 10.1038/nature02782
11. Noori HR. 2014. Hysteresis Phenomena in Biology. Springer.
12. Ogata N, Kozaki T, Yokoyama T, Hata T, and Iwabuchi K. 2015. Comparison between the Amount of Environmental Change and the Amount of Transcriptome Change. PloS one 10:e0144822. 10.1371/journal.pone.0144822
13. Ogata N, Yokoyama T, and Iwabuchi K. 2012. Transcriptome responses of insect fat body cells to tissue culture environment. PloS one 7:e34940. 10.1371/journal.pone.0034940
14. Seekaki P, and Ogata N. 2017. Calculating Kolmogorov Complexity from the Transcriptome Data. arXiv:1704.03559. https://arxiv.org/abs/1704.03559
15. Sun J, Nishiyama T, Shimizu K, and Kadota K. 2013. TCC: an R package for comparing tag count data with robust normalization strategies. BMC bioinformatics 14:219. 10.1186/1471-2105-14-219
16. Wang L, Zhu H, Wu J, Li N, and Hua J. 2014. Characterization of embryonic stem-like cells derived from HEK293T cells through miR302/367 expression and their potentiality to differentiate into germ-like cells. Cytotechnology 66:729-740. 10.1007/s10616-013-9639-2