博士（薬　学）　アシフ　モハマド　ザカリア

学 位 論 文 題 名

DEVELOPMENT OF GLYCOSPHINGOLIPID (GSL) GENE EXPRESSION ARRAY AND ITS APPLICATION

（スフィンゴ糖脂質合成酵素遺伝子アレイの開発とその応用）

学位論文内容の要旨

To identify the genes that are involved in the biosynthesis and metabolism of Glycosphingolipids (GSLs) two custom nylon membrane based gene expression arrays were developed for mouse and human genes. Glycosphingolipids and gangliosides are reported to be receptors for microorganisms and their toxins, modulators of cell growth and differentiation, and organizers of cellular attachment to matrices. Some GSLs are found to act as antigens for numerous antibodies. Aberrant expression of gangliosides can be correlated to different pathological conditions, such as insulin resistance or type-2 diabetes, and different types of cancers. Since the changes of GSL expression in various physiological and pathological conditions are affected by the combined actions of the biosynthesis and metabolism of GSLs, the development of expression profiling system for these GSL related genes would be a key technique in elucidating the role of these components.

The inability of the commercial gene expression profiling systems to detect GSL related genes either due to their low sensitivity towards this special group of genes or the extremely low expression of GSL genes requires a different approach for profiling the genes. Probes for the expression array were constructed from different cDNA libraries and cloned into pGEM-T easy vector. These plasmid templates were used as template for PCR amplification. Amplification using universal primers, such as M13 reverse/forward primers or T7/SP6 primers produced non-specific signals or false positives. In order to reduce non-specific signals the amplification was performed with gene specific primers using 1~5 ng of plasmid template. This improvement significantly reduced non-specific signals in the final results. The purified products were spotted onto positively charged Nylon membrane. Due to the very low expression of sphingolipid related genes, the optimum probe concentration was determined. The most reliable results were obtained using 50 and 25 ng per spot which was 10~100 folds higher than other arrays.

To evaluate the sensitivity and specificity of GSL gene expression array system, the gene expression profile of GM3 synthase (SAT-I) expressing cells was checked using GM3 reconstituted J5/SAT-I cell. J5/SAT-I which expresses higher level of GM3 ganglioside compared to mock transfected J5 cells also showed higher SAT-I mRNA expression. Real-Time PCR analysis confirmed the result obtained from GSL array.

Investigation of the differentiation of F9 in response to 3 ~ 4 days of retinoic acid

(RA) treatment showed upregulation of ganglioside content by almost 24 folds by thin layer chromatographic analysis. TLC analysis further revealed that b and c series gangliosides were also synthesized in response to retinoic acid, which prompted to speculate that several genes in the GSL biosynthetic pathway may also be expressed during differentiation. Analysis using custom GSL array showed a global expression of GSL related genes. The activity of GM3 synthase and GM3 content were increased during the differentiation of human promyelocytic leukemia HL-60 cells into the monocyte/macrophage lineage after 36 hours of treatment with PMA. Studies of the GSL gene expression profile of this cell confirmed the expression of several GSL related genes in differentiated HL-60 cell compared to undifferentiated cell.

The GSL synthase genes expression profile was analyzed using several Non-small lung cancer cells (NSCLC) whose GM3 expression was studied using Real-time PCR and TLC method. Real- time PCR analysis showed that the expression of hSAT-I mRNA in PC3 was twice than that of A549, and GM3 ganglioside content was significantly high which correlates with SAT-I RNA expression. On the other hand, the chemical content of GM3 ganglioside was almost absent in A549. Despite the low expression of SAT-I in A549, detectable level of GM3 ganglioside was expected. To answer this issue, the comparative GSL array analysis of PC3 and A549 was studied. GSL array analysis revealed that the expression levels of both cell lines were quite different. PC3 showed higher signal for SAT-I mRNA compared to A549, which reflects the results obtained from real-time PCR and TLC analysis. However, careful analysis also reveals higher expression of GalNAc transferase (GalNacT) or GM2/GD2 synthase RNA in A549 compared to PC3 by approximately 2 folds. Higher activity of GalNAcT indicates that a large fraction of GM3 ganglioside was converted to GM2 ganglioside by the activity of GalNAcT. This study demonstrates the usefulness of a biosynthetic pathway based expression profiling systems such as our GSL array in finding defects in a gene expression which may lead to alteration of expression of subsequent products.

Custom Array system such as our GSL array can be constructed to detect low expressing genes which are otherwise undetected by commercial arrays. This study also demonstrates the usefulness of custom array such our GSL gene expression array in investigating a particular biological pathway like the glycosphingolipid biosynthesis pathway. From this study it is apparent that cell differentiation processes including retinoic acid treated F9 embryonic carcinoma and PMA treated HL-60 leukemia cells exhibit global alteration of GSL biosynthesis and metabolism. GSL array can help to grasp the entire picture on the regulation of GSL biosynthesis and metabolism in different biological processes such as cell differentiation, proliferation, apoptosis or pathological conditions such as cancer, diabetes, etc. The investigation of non-small cell cancer lines – A549 and PC3 shows that using a gene expression profile which can analyze a particular biological pathway can help pinpoint one or more specific genes responsible for the conversion of a substrate(s) to the subsequent product.

学位論文審査の要旨

主　査　助教授　井ノ口　仁　一
副　査　教　授　五十嵐　靖　之
副　査　教　授　有　賀　寛　芳
副　査　助教授　松　本　健　一

学位論文題名

DEVELOPMENT OF GLYCOSPHINGOLIPID (GSL) GENE EXPRESSION ARRAY AND ITS APPLICATION

(スフィンゴ糖脂質合成酵素遺伝子アレイの開発とその応用)

アシフ モハマド ザカリア君はスフィンゴ糖脂質合成酵素遺伝子アレイ（GSL cDNA アレイ）の開発とその応用に関して，下記に要約される研究を行った。

スフィンゴ糖脂質 (GSL) は長鎖のアミノアルコールであるスフィンゴシンと長鎖脂肪酸からなるセラミドに種々の糖が結合して生合成される。GSLは生体膜の主要な構成糖脂質であり，細胞膜上でラフトと呼ばれる微細なドメインを形成し，細胞間のシグナル伝達や細胞内物質輸送において重要な役割を果たすことが次々と明らかになってきている。また，GSLが哺乳動物の生存に必須であることや細胞内シグナル伝達制御物質としての機能を持つことが明らかになり，近年その重要性が注目を集めている。しかしながら，その制御のメカニズムや役割については十分解明されているとは言いがたい。GSLの糖鎖の発現制御機構を解明していく目的において，糖鎖そのものの変化だけでなく，糖鎖合成遺伝子（糖転移酵素遺伝子）および，糖鎖と相互作用する分子の変化をも含めて検討していくことが重要であり，網羅的な研究手法が必要であると考えられる。

本研究では，GSLの発現に関わる遺伝子群の解析を中心に研究を進めGSL発現に関わる多数の遺伝子発現を同時に解析することで，生体での糖鎖発現制御の仕組みを明らかにすることを目指している。すでに、市販のマイクロアレイではGSL関連遺伝子の定量的な検出はそれらの遺伝子が非常に低発現であるため困難であることが判明していた。従って，本研究の第一の目的は迅速かつ簡便にGSL関連遺伝子の発現が定量できる系 (GSL cDNAアレイ)を確立することを目的とした。ヒトおよびマウスを対象とし，糖転移酵素

や糖鎖分解酵素，および糖修飾酵素などの直接に糖鎖の合成に関与する遺伝子群のほか，糖脂質などの糖鎖修飾を受けるコア構造に関する遺伝子群，またスフィンゴ脂質，リン脂質などの糖鎖関連分子群に関与する遺伝子群などおよそ40遺伝子にのぼる遺伝子を選定し，試行錯誤の結果，定量的GSL cDNAアレイの開発に成功した。

次に，レチノイン酸 (RA) によるマウス奇形種細胞株 F9 の始原内胚葉への分化に伴うガングリオシド合成酵素の発現変化に注目して検討を行った。マウス GSL cDNA アレイを用いて F9 未分化細胞と RA により始原内胚葉へ分化した細胞の解析から，分化過程において GSL の代謝経路に直接関与する２９の酵素のうち，２７もの酵素の遺伝子レベルが上昇していることが確認された。従って，GSL の生合成に関与する遺伝子群のうち９０％以上もの遺伝子が分化に伴って上昇したことを初めて証明することができたと評価される。また，ヒトの GSL cDNA アレイの特異性と感受性を HL-60 骨髄球白血病細胞の分化誘導系において証明している。

これらの研究により，GSL の発現調節機構の解明には，生合成と代謝に関る遺伝子発現を正確に把握することが重要であり，GSL cDNA アレイは基礎および臨床研究に非常に有用であると評価し，学位論文として充分価値を有するものと評価する。