マイクロアレイを用いた環境化学物質代謝に関与する遺伝子多型判定法の開発と問題点

Ban, Susumu
Department of Public Health, Hokkaido University, Graduate School of Medicine

Kondo, Tomoko
Department of Public Health, Hokkaido University, Graduate School of Medicine

Ishizuka, Mayumi
Laboratory of Toxicology, Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University

Sasaki, Seiko
Department of Public Health, Hokkaido University, Graduate School of Medicine

他

https://doi.org/10.15017/6140

出版情報：福岡醫學雜誌. 98 (5), pp.208-214, 2007-05-25. Fukuoka Medical Association
バージョン：
権利関係：

Using Microarray Analysis to Evaluate Genetic Polymorphisms Involved in the Metabolism of Environmental Chemicals

Susumu BAN[1], Tomoko KONDO[1], Mayumi ISHIZUKA[2], Seiko SASAKI[1], Kanae KONISHI[1], Noriaki WASHINO[1], Syoichi FUJITA[2] and Reiko KISHI[1]

[1] *Department of Public Health, Hokkaido University Graduate School of Medicine,*
[2] *Labratory of Toxicology, Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Japan.*

Abstract The field of molecular biology currently faces the need for a comprehensive method of evaluating individual differences derived from genetic variation in the form of single nucleotide polymorphisms (SNPs). SNPs in human genes are generally considered to be very useful in determining inherited genetic disorders, susceptibility to certain diseases, and cancer predisposition. Quick and accurate discrimination of SNPs is the key characteristic of technology used in DNA diagnostics. For this study, we first developed a DNA microarray and then evaluated its efficacy by determining the detection ability and validity of this method. Using DNA obtained from 380 pregnant Japanese women, we examined 13 polymorphisms of 9 genes, which are associated with the metabolism of environmental chemical compounds found in high frequency among Japanese populations. The ability to detect *CYP1A1* I462V, *CYP1B1* L432V, *GSTP1* I105V and *AhR* R554K gene polymorphisms was above 98%, and agreement rates when compared with real time PCR analysis methods (kappa values) showed high validity: 0.98 (0.96), 0.97 (0.93), 0.90 (0.81), 0.90 (0.91), respectively. While this DNA microarray analysis should prove important as a method for initial screening, it is still necessary that we find better methods for improving the detection of other gene polymorphisms not part of this study.

Introduction

The field of molecular biology currently faces the need for a comprehensive method of evaluating individual differences derived from genetic variation in the form of single nucleotide polymorphisms (SNPs). SNPs in human genes are generally considered to be very useful in determining inherited genetic disorders, susceptibility to certain diseases, and cancer predisposition.

Quick and accurate discrimination of SNPs is the key characteristic of technology used in DNA diagnostics[1]. While PCR-RFLP analysis and real-time PCR analysis have been the standard methods for the discrimination of SNPs[2)3)], it is impossible to simultaneously discriminate multiple SNPs using PCR-RFLP analysis, while real-time PCR analysis requires access to an expensive device. Since multiple genetic factors tend to play a part in increased susceptibility to diseases related to chemical compounds, it is important to develop a method that allows for the quick discrimination of multiple SNPs simultaneously and without the use of expensive machinery. DNA microarrays are powerful tools for genomic analysis and enable one to discriminate many SNPs simultaneously[4)~6)]. However, DNA microarray methods used today remain expensive since a special device for

Address correspondence and requests for reprints to:
Dr. Susumu BAN
Department of Public Health, Hokkaido University Graduate School of Medicine, Kita 15, Nishi 7, Kita-ku, Sapporo 060-8638, Japan.
E-mail: sban@med.hokudai.ac.jp
Phone; +81-11-706-5068 Fax: +81-11-706-7805

measuring fluorescence signals is required. Therefore, we developed a new DNA microarray method for quick and accurate discrimination of SNPs at a lower cost.

We selected 13 polymorphisms of 9 genes (Cytochrome P450*(CYP)1A1* I462V, *CYP1A2*-3860A>G, *CYP1B1* A119S and L432V, *CYP2A6* deletion and I471T, *CYP2C19* 636A>G and 681G>A, *CYP2D6* P34S, *GSTP1* I105V, *NAT2* R197Q and G286E and *AhR* R554K), which are found in high frequency among Japanese populations. Environmental chemical compounds are considered to be the cause of harmful influences such as male congenital anomalies (cryptorchidism, hypospadias, etc.), endocrine disruption, and child motor development[7)~9)]. CYP enzymes, the GST family, and the Ah receptor (AhR) are all elements influencing individual susceptibility to environmental chemical compounds[10)11)].

In this study, we examined the usefulness of this array by determining the accuracy and ability to detect genetic polymorphisms using DNA from 380 pregnant Japanese women.

Material and Methods

Study population

A prospective cohort study was conducted between 2002 and 2004 in Sapporo, Japan (*the Hokkaido Study on Environment and Children's Health*). In this study, the subjects comprised 380 pregnant women who enrolled at 23-35 weeks of gestation and successfully delivered single, live births at the Sapporo Toho Hospital. The study was conducted with the informed consent of all subjects and was approved by the Institutional Ethical Board for Human Gene and Genome Studies at Hokkaido University's Graduate School of Medicine.

DNA sample

Peripheral blood samples were collected at the time of enrollment in the study. Genomic DNA was extracted from the peripheral blood sample by standard techniques[12)].

DNA Microarray

We selected 26 alleles from 9 genes: [*CYP1A1* I462V (X02612), *CYP1A2*-3860A>G (dbSNP ID: rs2069514), *CYP1B1* A119S and L432V (U56438), *CYP2A6* deletion and I471T (U22027), *CYP2C19* 636A>G and 681G>A (NT_030059), *CYP2D6* P34S (M33189), *GSTP1* I105V (M24485), *NAT2* R197Q and G286E (X14672) and *AhR* R554K (dbSNP ID: rs2066853)], all of which may be influenced by the metabolism of certain environmental chemicals (Table 1). In

Table 1 Polymorphisms of genes on the glass array

Gene	Accession No.	Polymorphism	Mutation Position	Base
CYP1A1	X02612	① I462V	6819	A/G
CYP1A2	dbSNP ID: rs2069514	②−3860A>G	−3860	G/A
CYP1B1	U56438	③ A119S	4160	G/T
		④ L432V	8131	C/G
CYP2A6	U22027	⑤ Deletion		
		⑥ I471T	6416	T/C
CYP2C19	NT_030059	⑦ 636A>G	180546	G/A
		⑧ 681G>A	1806252	G/A
CYP2D6	M33189	⑨ P34S	913	C/T
GSTP1	M24485	⑩ I105V	2627	A/G
NAT2	X14672	⑪R197Q	1311	G/A
		⑫G286E	1578	G/A
AhR	dbSNP ID: rs2066853	⑬R554K	181	A/G

cooperation with Nisshinbo, we developed a glass array, to which the oligonucleotides of 26 alleles of 9 genes were tethered.

Amplification of 13 fragments from the genomic region containing the mutation positions was performed in a multiplex PCR assay in a 50-μL volume containing 100 ng of genomic DNA, 50 pmol each of multiplex PCR primer and QIAGEN multiplex PCR master mix (QIAGEN). Reactions were carried out in the GeneAmp PCR System 9700 (Applied Biosystems) according to the following condition : 95°C for 15 minutes, 40 cycles of 95°C for 1 minute, 60°C for 30 seconds, 72°C for 30 seconds, and 72°C for 3 minutes. After the multiplex PCR assay, the PCR products were then treated at 95°C for 1 minute, on ice for 1 minute, followed by the addition of 10 μL of hybridization buffer. A glass array was hybridized with the reaction solution for 2 hours at 37°C. After hybridization, the glass array was washed with the wash buffer for 5 minutes and put in the conjugate buffer at room temperature for 30 minutes. After washing, the glass array was treated with the coloring buffer at room temperature for 30 minutes. After the drying, the glass array was scanned and gene polymorphisms were discerned.

Real-time PCR

CYP1A1 I462V, *CYP1B1* L432V, *GSTP1* I105V and *AhR* R554K polymorphisms were genotyped by allelic discrimination using fluorogenic probes and the 5' nuclease (TaqMan®) assay according to the manufacturer's protocol. SNP genotyping products were used as probes (Applied Biosystems, Foster City, USA). For the *CYP1B1* L432V, *GSTP1* I105V and *AhR* R554K polymorphisms, we purchased the TaqMan® SNP genotyping product c_3099976_30 (dbSNP : rs1056836), c_3237198_1_(dbSNP : rs947894) and c_11170747_10 (dbSNP : rs2066853), respectively. We used Custom TaqMan® Genomic Assays for the *CYP1A1* I462V (dbSNP : rs1048943) and the primers were designed as follows : forward, 5'-ATGGGCAAGCGGAAGTGTA ; reverse, 3'-CAGGATAGCCAGGAAGAGAAAGAC. PCR was performed in 10 μl, with approximately 40 ng of genomic DNA, 0.25 μl 40 × Assays-by-Design SNP Genotyping Assay Mix or 0.5 μl 20 × Assays-on-Demand SNP Genotyping Assay Mix (consisting of unlabeled PCR primers and TaqMan® MGB probe, FAM and VIC dye-labeled) and 5.0 μl of 2 × TaqMan® Universal PCR Master Mixture. Cycling conditions were 2 minutes at 50 °C, followed by 10 minutes at 95 °C and then 40 cycles of 92 °C for 15 seconds and 60 °C for 1 minute. PCR products were measured at 490/520 nm excitation/emission (FAM) and 530/560 nm excitation/emission (VIC) at 60 °C for 1 minute and genotyped using allelic discrimination with the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions.

Results and discussion

The discrimination results of the *CYP1A1* I462V, *CYP1B1* L432V, *GSTP1* I105V and *AhR* R554K for 380 pregnant women are summarized in Table 2. The rates of discrimination of *CYP1A1* I462V, *CYP1B1* L432V, *GSTP1* I105V and *AhR* R554K gene polymorphisms by the microarray were 100%, 100%, 98.4% and 99.7%, respectively, which demonstrates high detection rates for these gene polymorphisms. On the other hand, we could not determine other polymorphisms taking place in genes other than the four genes mentioned above.

Next, we compared the results of the four gene polymorphisms by microarray with the results of real time PCR analysis, which is considered to be a highly reliable method. We calculated the agreement rate and kappa values to estimate the validity of the microarray method and the real-time PCR method for *CYP1A1* I462V, *CYP1B1* L432V, *GSTP1* I105V and *AhR* R554K polymorphisms. Agreement rates (kappa values) of four gene polymorphisms were 0.98 (0.96), 0.97 (0.93), 0.90 (0.81), 0.90 (0.91), respectively (Table 3).

The microarray system was then used to evaluate the susceptibility to specific diseases or to determine compounds influencing human genes[13)14)]. In this study, we developed a glass array to evaluate the susceptibility to environmental chemical compounds commonly found in Japanese populations. For the *CYP1A1* I462V, *CYP1B1* L432V, *GSTP1* I105V and *AhR* R554K gene polymorphisms, the microarray analysis showed high detection power (above 98%) and the discrimination results by this microarray analysis compared with the results by the real-time PCR method shows very good validity. The results indicate that our microarray method is useful for initial screenings meant to detect *CYP1A1* I462V, *CYP1B1* L432V, *GSTP1* I105V and *AhR* R554K gene polymorphisms.

As for gene polymorphisms other than the *CYP1A1* I462V, *CYP1B1* L432V, *GSTP1* I105V and *AhR* R554K gene polymorphisms, signals were obtained, but at similar levels for both alleles. Therefore, we could not determine the likelihood of gene polymorphism. This may be due to hybridization conditions. However, the efficiency of hybridization and the thermal stability of hybrids between the target nucleic acid on the glass array and the probes is strongly sequence dependent. Other microarray approaches such as minisequencing[15)16)], solid-phase primer elongation[17)18)], or solid-phase PCR[19)20)], utilize the fidelity of DNA polymerase enzymes, but require a fluorescence scanning system. In general, DNA extension reaction by DNA polymerase uses an unlabeled target. Therefore an improved assay will be required to discriminate other gene polymorphisms quickly and at a low cost.

In conclusion, the microarray method used in this study has demonstrated good detection and high validity rates, and thus is appropriate for use in to initial screenings for some gene polymorphisms such as *CYP1A1* I462V, *CYP1B1* L432V, *GSTP1* I105V and *AhR* R554K. The ability to

Table 2 Determination of *CYP1A1* I462V, *CYP1B1* L432V, *GSTP1* I105V and *AhR* R554K polymorphisms in 380 pregnant women by microarray method

CYP1A1 I462V		*CYP1B1* L432V	
Genotype	n (%)	Genotype	n (%)
Ile/Ile	233 (61.3)	Leu/Leu	286 (75.2)
Ile/Val	132 (34.7)	Leu/Val	86 (22.6)
Val/Val	15 (3.9)	Val/Val	8 (2.1)
undetermined	none	undetermined	none
GSTP1 I105V		*AhR* R554K	
Genotype	n (%)	Genotype	n (%)
Ile/Ile	274 (72.1)	Arg/Arg	148 (38.9)
Ile/Val	96 (25.3)	Arg/Lys	156 (41.1)
Val/Val	4 (1.1)	Lys/Lys	75 (19.7)
undetermined	6 (1.6)	undetermined	1 (0.3)

Table 3 Validity of the microarray method for *CYP1A1* I462V, *CYP1B1* L432V, *GSTP1* I105V and *AhR* R554K polymorphisms in 380 pregnant women

CYP1A1 I462V

Results of Real-time PCR	Results of Microarray			
	I/I	I/V	V/V	
I/I	230	3	1	234
I/V	3	128	0	131
V/V	0	1	14	15
	233	132	15	380

CYP1B1 L432V

Results of Real-time PCR	Results of Microarray			
	L/L	L/V	V/V	
L/L	282	8	0	290
L/V	4	78	0	82
V/V	0	0	8	8
	286	86	8	380

GSTP1 I105V

Results of Real-time PCR	Results of Microarray			
	I/I	I/V	V/V	
I/I	254	12	0	266
I/V	20	83	0	103
V/V	0	1	4	5
	274	96	4	374

AhR R554K

Results of Real-time PCR	Results of Microarray			
	I/I	I/V	V/V	
I/I	121	0	1	122
I/V	27	148	0	175
V/V	0	8	74	82
	148	156	75	379

Gene polymorphism	Agreement rate*1	Kappa value*2	P value
CYP1A1 I462V	0.98	0.96	<0.001
CYP1B1 L432V	0.97	0.93	<0.001
GSTP1 I105V	0.90	0.81	<0.001
AhR R554K	0.90	0.91	<0.001

* 1 : Agreement rate = X/Y
(X : Number of samples which have same discrimination results for both microarray and real-time PCR tests, Y : Number of determinable samples by microarray analysis)
* 2 : Kappa value = O-E/1-E
(O : Rate of the same results for both microarray and real-time PCR tests, E : Expected value of the same results for both microarray and real-time PCR tests)

discriminate multiple polymorphisms using this microarray analysis allows for convenient, quick, and low-cost estimation of susceptibility to harmful influences and disease stemming from environmental chemical compounds commonly found in Japanese populations.

Acknowledgements

This study was supported in part by a Grant-in-Aid for Health Scientific Research from the Japan Ministry of Health, Labor, and Welfare, and by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science.

References

1) Graber JH, O'Donnel MJ, Smith CL and Cantor CR : Advances in DNA diagnostics. Curr. Opin. Biotechnol. 9 : 14-18, 1998.
2) Haliassos A, Chomel JC, Tesson L, Baudis M, Kruh J, Kaplan JC and Kitzis A : Modification of enzymatically amplified DNA for the detection of point mutations. Nucleic Acids Res. 17 : 3606, 1989.
3) Kostrikis LG, Tyagi S, Mhlanga MM, Ho DD and Kramer FR : Spectral genotyping of human alleles. Science 279 : 1228-1229, 1998.
4) Landegren U, Nilsson M and Kwok PY : Regarding bits of genetic information : Methods for single-nucleotide polymorphism analysis. Genome Res. 8 : 769-776, 1998.

5) Wang J : From DNA biosensors to gene chips. Nucleic Acids Res. 28 : 3011–3016, 2000.
6) Tillib SV and Mirzabekov AD : Advances in the analysis of DNA sequence variations using oligonucleotide microchip technology. Curr. Opin. Biotechnol. 12 : 53–58, 2001.
7) Vidaeff AC and Sever LE : In utero exposure to environmental estrogens and male reproductive health : a systematic review of biological and epidemiologic evidence. Reprod. Toxicol. 20 : 5–20, 2005.
8) Steinhardt GF : Endocrine disruption and hypospadias. Adv. Exp. Med. Biol. 545 : 203–215, 2004.
9) Nakajima S, Saijo Y, Kato S, Sasaki S, Uno A, Kanagami N, Hirakawa H, Hori T, Tobiishi K, Todaka T, Nakamura Y, Yanagiya S, Sengoku Y, Iida T, Sata F and Kishi R : Effects of prenatal exposure to polychlorinated biphenyls and dioxins on mental and motor development in Japanese children at 6 months of age. Environ. Health Perspect. 114 : 773–778, 2006.
10) Thier R, Bruning T, Roos PH, Rihs HP, Golka K, Ko Y and Bolt HM : Markers of genetic susceptibility in human environmental hygiene and toxicology : the role of selected CYP, NAT and GST genes. Int. J. Hyg. Environ. Health 206 : 149–171, 2003.
11) Hung RJ, Boffetta P, Brennan P, Malaveille C, Hautefeuille A, Donato F, Gelatti U, Spaliviero M, Placidi D, Carta A, Scotto di Carlo A and Porru S : GST, NAT, SULT1A1, CYP1B1 genetic polymorphisms, interactions with environmental exposures and bladder cancer risk in a high-risk population. Int. J. Cancer 110 : 598–604, 2004.
12) Saijo Y, Sata F, Yamada H, Suzuki K, Sasaki S, Kondo T, Gong YY, Kato EH, Shimada S, Morikawa M, Minakami H and Kishi R : Ah receptor, CYP1A1, CYP1A2 and CYP1B1 gene polymorphisms are not involved in the risk of recurrent pregnancy loss. Mol. Hum. Reprod. 10 : 729–733, 2004.
13) Welsh JB, Zarrinkar PP, Sapinoso LM, Kern SG, Behling CA, Monk BJ, Lockhart DJ, Burger RA and Hampton GM : Analysis of gene expression profiles in normal and neoplastic ovarian tissue samples identifies candidate molecular markers of epithelial ovarian cancer. Proc. Natl. Acad. Sci. USA 98 : 1176–1181, 2001.
14) Francois E, Wang DY, Fulthorpe R, Liss SN and Edwards EA : DNA microarrays for detecting endocrine-disrupting compounds. Biotechnol. Adv. 22 : 17–26, 2006.
15) Pastinen T, Kurg A, Metspalu A, Peltonen L and Syvanen AC : Minisequencing : A specific tool for DNA analysis and diagnostics on oligonucleotide arrays. Genome Res. 7 : 606–614, 1997.
16) Dubiley S, Kirillov E and Mirzabekov A : Polymorphism analysis and gene detection by minisequencing on an array of gel-immobilized primers. Nucleic Acids Res. 27 : e19, 1999.
17) Pastinen T, Raitio M, Lindroos K, Tainola P, Peltonen L and Syvanen AC : A system for specific, high-throughput genotyping by allelic-specific primer extension on microarrays. Genome Res. 10 : 1031–1042, 2000.
18) Erdogan F, Kirchner R, Mann W, Ropers HH and Nuber UA : Detection of mitochondrial single nucleotide polymorphisms using a primer elongation reaction on oligonucleotide microarrays. Nucleic Acids Res. 29 : e36, 2001.
19) Strizhkov BN, Drobyshev AL, Mikhailovich VM and Mirzabekov AD : PCR amplification on a microarray of gel-immobilized oligonucleotides : Detection of bacterial toxin- and drug-resistant genes and their mutations. Biotechniques 29 : 844–850, 2000.
20) Tillib SV, Strizhkov BN and Mirzabekov AD : Integration of multipole PCR amplifications and DNA mutation analyses by using oligonucleotide microchip.Anal. Biochem. 292 : 155–160, 2001.

(Received for publication April 11, 2007)

(和文抄録)

マイクロアレイを用いた環境化学物質代謝に関与する遺伝子多型判定法の開発と問題点

[1)] 北海道大学大学院医学研究科 予防医学講座 公衆衛生学分野
[2)] 北海道大学大学院獣医学研究科 環境獣医学講座 毒性学教室

坂　　晋[1)]，近藤朋子[1)]，石塚真由美[2)]，佐々木成子[1)]，小西香苗[1)]，
鷲野考揚[1)]，藤田正一[2)]，岸　玲子[1)]

近年，一遺伝子多型(SNPs)に由来する個々の感受性を包括的に評価する方法が必要とされている．ヒト遺伝子における SNP は，遺伝的な疾患，特定の疾病に対する感受性，癌などの素因のひとつとして考えられている．そのため，迅速で正確な SNPs 判定法が求められている．本研究では，環境化学物質代謝に関与する遺伝子多型を判定するための DNA マイクロアレイを開発し，その効力と信頼性を 380 人の日本人妊婦の血液を用いて，日本人で変異型頻度の高い 9 遺伝子 13 多型について検討した．CYP1A1 I462V，CYP1B1 L432V，GSTP1 I105V，AhR R554K 遺伝子多型に対する検出力は 98%以上の値を示すとともに，リアルタイム PCR 法による結果との比較による信頼度 (κ 係数) は，高い値を示した (それぞれ，0.98 (0.96)，0.97 (0.93)，0.90 (0.81)，0.90 (0.91))．本研究のマイクロアレイ法は，検出できなかった遺伝子多型について改善する必要がある一方で，初期のスクリーニング方法として重要である．