ELSEVIER

CYTOKINE

www.elsevier.com/locate/jnlabr/ycyto

Cytokine 27 (2004) 135–141

Genotyping of single nucleotide polymorphisms in cytokine genes using real-time PCR allelic discrimination technology☆

Victor J. Johnson*, Berran Yucesoy, Michael I. Luster

Toxicology and Molecular Biology Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention, Morgantown, WV 26505, USA

Received 16 November 2003; received in revised form 20 April 2004; accepted 20 May 2004

Abstract

Single nucleotide polymorphisms (SNPs), particularly those within regulatory regions of genes that code for cytokines often impact expression levels and can be disease modifiers. Investigating associations between cytokine genotype and disease outcome provides valuable insight into disease etiology and potential therapeutic intervention. Traditionally, genotyping for cytokine SNPs has been conducted using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), a low throughput technique not amenable for use in large-scale cytokine SNP association studies. Recently, Taqman® real-time PCR chemistry has been adapted for use in allelic discrimination assays. The present study validated the accuracy and utility of real-time PCR technology for a number of commonly studied cytokine polymorphisms known to influence chronic inflammatory diseases. We show that this technique is amenable to high-throughput genotyping and overcomes many of the problematic features associated with PCR-RFLP including post-PCR manipulation, non-standardized assay conditions, manual allelic identification and false allelic identification due to incomplete enzyme digestion. The real-time PCR assays are highly accurate with an error rate in the present study of <1% and concordance rate with PCR-RFLP genotyping of 99.4%. The public databases of cytokine polymorphisms and validated genotyping assays highlighted in the present study will greatly benefit this important field of research.
Published by Elsevier Ltd.

Keywords: Polymorphism; Cytokine; Single nucleotide polymorphism (SNP); Real-time PCR; Taqman

1. Introduction

Examination of the entire human genome has revealed that approximately 99.9% of the DNA sequence is identical between any two randomly selected individuals. The other 0.1% represents polymorphic regions that differ between individuals and occurs at a frequency of at least 1% [1]. Single nucleotide polymorphisms (SNPs), consisting of the substitution of a single nucleotide pair, are the most abundant form of genetic variation occurring with a frequency of approximately 1 per 1000 base pairs [2]. SNPs do not, by themselves, cause disease but could predispose humans to disease, modify the extent or severity of the disease or influence drug response and treatment efficacy [3]. Evolutionary stability is a key feature of SNPs making them easier to follow in population studies and thus, they serve as excellent biological markers for constructing chromosome maps and pinpointing disease loci on the human genome map [4,5].

Both genetic and environmental factors have roles in the development of human disease. Genetic disorders are caused by abnormalities in an individual's genetic material. Monogenic diseases are caused by changes in the DNA sequence of a single gene. They are inherited in recognizable patterns and identified by family studies. On the other hand, polygenic diseases are caused by complex interactions among multiple genes as well as

☆ Mention of any company name or product does not constitute endorsement by the National Institute for Occupational Safety and Health (NIOSH).

* Corresponding author. Toxicology and Molecular Biology Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, 1095 Willowdale Road, Mail Stop 3014, Morgantown, WV 26505-2888 USA. Tel.: +1-304-285-6249; fax: +1-304-285-5708.

E-mail address: vjohnson3@cdc.gov (V.J. Johnson).

1043-4666/$ - see front matter Published by Elsevier Ltd.
doi:10.1016/j.cyto.2004.05.002

environmental and lifestyle factors. These diseases are more complicated than monogenic or chromosomal diseases. Some of the most common chronic diseases such as arthritis, Alzheimer's disease, coronary heart disease, diabetes, and cancer are under the control of many genes of modest individual effect and many non-genetic factors all of which interact to define disease phenotype.

Genetic association studies are designed to compare the frequencies of the alleles or genotypes at the site of interest in populations of cases and controls, with the expectation that disease-susceptibility variants will be associated with increased risk of disease. For complex disorders, genetic association studies should test the entire linkage disequilibrium pattern of candidate genes [6]. This will facilitate the identification of possible gene–gene interactions and other positional candidate genes from linkage studies [7]. Therefore, population studies designed to identify these variants require large numbers of subjects to achieve statistical power. Statistical estimations show that to detect odds ratio ≥ 1.5 with 80% power for an allele frequency of 10%, the case–control population size needs to exceed 700 patients [8,9]. A major challenge confronting research on disease genetics is the need to develop accurate, fast and inexpensive high-throughput genotyping techniques that will allow screening of large numbers of SNPs in large case–control populations. Large population sizes are both cost and time prohibitive to traditional sequencing and RFLP based methodologies especially when multiple SNPs are being investigated. Recent advances in molecular biology have led to the development of several new non-gel based high-throughput techniques such as allele-specific extension assay, allele-specific oligonucleotide ligations assay, Taqman®, molecular beacons, invader assay multilocus PCR, and array base techniques [10–12] for SNP genotyping amenable to large population sizes.

SNPs in the regulatory regions of cytokine genes affect expression levels and represent modifiers for a variety of common diseases including asthma, autoimmune diseases, periodontal diseases, diabetes, Alzheimer's disease, and coronary artery disease [13]. Due to the ever-increasing number of functionally relevant SNP-markers in cytokine genes and to the large sample size necessary for association studies, an economical and high-throughput genotyping technology is required. In the present study, real-time PCR (Taqman®) genotyping assays were designed and validated for several important functional cytokine polymorphisms in *IL-1*, *IL-10*, *TNFα* and *TGF-β* genes which have been shown to be associated with many immune and inflammatory diseases. The data presented demonstrate the utility of this technique for cytokine polymorphism analysis. The results from real-time PCR analysis were validated by comparison to PCR-RFLP technique, considered the "gold standard", on the same genomic DNA samples. Our data support the use of real-time PCR genotyping as a high-throughput technique in future studies investigating cytokine polymorphisms in disease susceptibility and severity. We have provided validated assay conditions (primers, probes and PCR parameters) for eight important cytokine SNPs and the databases identified in this manuscript provide validated assays for a multitude of additional cytokine (and other genes) SNPs.

2. Results and discussion

All of the cytokine SNPs tested in the present study were in Hardy–Weinberg equilibrium (Table 1). In addition, all of the real-time PCR assays used in the present study have been validated by direct sequencing by the SNP500Cancer group at the National Cancer Institute (http://snp500cancer.nci.nih.gov/home.cfm). The purpose of the present study was to evaluate the utility and accuracy of real-time PCR allelic discrimination assays for cytokine genotyping of human genomic DNA samples against the more conventional PCR-RFLP technique. To achieve this goal, we genotyped 100 independent DNA samples using real-time PCR and PCR-RFLP assays for each cytokine SNP. Based on genotyping results for 800 independent DNA samples by real-time PCR and PCR-RFLP, the calculated concordance rate was 99.4% (5 mismatches/800 genotypes). Re-analysis of the mismatching genotypes confirmed that the original real-time PCR result was correct. Inspection of the original RFLP gel results showed that discrepant samples produced poor restriction patterns thus accounting for the mis-identification. An additional 65 independent DNA samples were genotyped in duplicate (two separate assays) by real-time PCR giving an error rate of $<1\%$ for this technique (Table 1). The error rate for PCR/RFLP in this study was 3.2% based on 125 samples genotyped in duplicate (data not shown). The increased accuracy of

Table 1
Comparison of genotype calls assayed by real-time PCR and PCR-RFLP

Cytokine SNP	Hardy–Weinberg	Taqman® vs. Taqman®	Taqman® vs. RFLP
TNFα(−238)	Pass	0/65[a]	0/100
TNFα(−308)	Pass	0/65	0/100
IL-1β(−511)	Pass	2/65	4/100
IL-1β(+3953)	Pass	0/65	1/100
IL-1α(+4845)	Pass	0/65	0/100
TGFβ1(−509)	Pass	3/65	0/100
IL-10(−819)	Pass	0/65	0/100
IL-10(−1082)	Pass	0/65	0/100
Error rate (%)		0.96	0.63

[a] Errors/total genotypes.

real-time PCR genotyping will contribute to increased power in genetic association studies by reducing misclassification and bias towards the null hypothesis. Examples showing the separation of genotypes obtained using real-time PCR assays are shown in Fig. 1. It is evident from the bivariate plots that this technique provides robust separation of the genotype clusters. The probe for each allele is labeled with a different fluorophore (6FAM or VIC) thus producing fluorescence at two separate wavelengths. Genotypes are then identified based on fluorescence clustering patterns in a bivariate plot. Samples that fall outside the clusters or that are close to the no-template controls (lower left clusters) are repeated, part of routine quality control procedures in addition to a random 10% repeat.

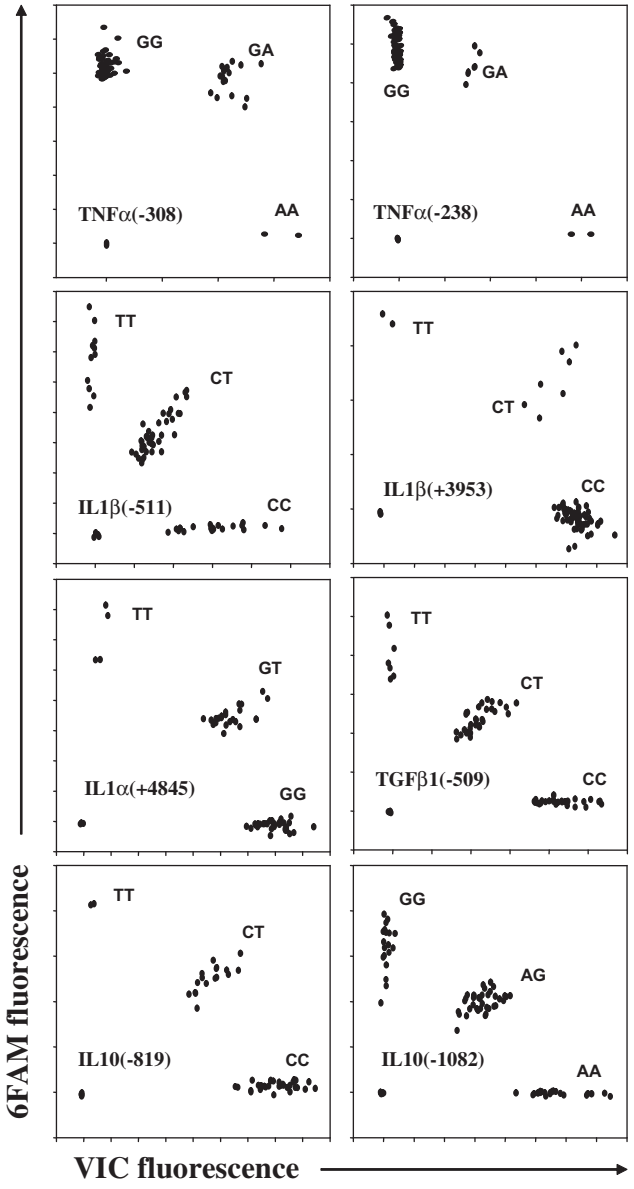

Fig. 1. Two parameter amplification plots demonstrating the separation of the three genotype populations and no template controls (lower left population) for each real-time PCR assay examined in this study including *TNFα*(−238), *TNFα*(−308), *IL-1β*(−511), *IL-1β*(+3953), *IL-1α*(+4845), *TGFβ1*(−509), *IL-10*(−819) and *IL-10*(−1082). Genomic DNA was amplified using standardized PCR conditions and fluorescence detection was performed using an iCycler® real-time instrument. Probe and primer sets are given in Table 2. Data for 90 genotypes per assay are shown.

Assay design and validation is often the most time consuming and labor intensive aspect of a SNP genotyping project. Table 2 compiles the probe/primer sets for each SNP assay and identifies the allele associated with each probe. These assays are designed to perform under universal conditions for probe/primer concentration, cycling temperatures, number of cycles, enzyme concentration and PCR buffer composition. Universal conditions are essential for high-throughput multi-SNP genotyping projects using large subject bases as they facilitate sample preparation and reduce opportunities for calculation errors in both reagent preparation and the setup of cycling conditions. Our laboratory has designed over 20 real-time PCR assays all of which amplify robustly using the universal conditions. In contrast, universal conditions cannot be applied to PCR-RFLP assays.

Incomplete enzyme digestion is a problem inherent to the PCR-RFLP technique for genotyping. The *TGFβ1*(−509) cytokine SNP can serve as an example to elaborate this issue. The digestion products for the CC, CT and TT genotypes are 429 + 26, 455 + 429 + 26 and 455 bp, respectively as illustrated in Fig. 2 (26 bp band is not visible). Incomplete digestion of a CC genotype will result in the appearance of bands at 455, 429 and 26 bp resulting in a false positive for the heterozygous CT genotype. The RFLP products shown in Fig. 2a are the result of digestion with 10 U *Eco* 81 I at 37 °C for 18 h. The same samples digested with 2-fold more enzyme (Fig. 2b) clearly demonstrate the errant identification of the CC genotypes as heterozygous due to incomplete digestion. Similar findings have been published previously for the *IL-6*(−174) cytokine SNP and the authors suggested that a non-mutation dependent cleavage site be introduced into one primer to monitor completion of digestion [14]. Incomplete digestion can conceivably contribute to errors in samples that yield greater amounts of PCR product. Such samples may require additional enzyme or time to fully digest indicating that optimization of enzyme concentration and digestion time is critical to the success of PCR-RFLP based genotyping. This problem is overcome with the real-time PCR allelic discrimination assays since the technique does not rely on any post-PCR manipulations. Error rate using the real-time PCR method was <1%, whereas the error rate for PCR-RFLP was 3.2% [based on 125 samples genotyped in duplicate for *IL1 receptor antagonist* (+2018) SNP]. Error rates for the two techniques used in this study were not statistically

Table 2
Cytokine polymorphism real-time PCR probe/primer combinations used in this study

Cytokine SNP	Substitution	Oligo	Sequence 5′ → 3′ (fluorophore/quencher)
TNFα(−238)[a]	A→G	Probe-A	VIC-**CTCCCTGCTCTGATTC**-MGB[b]
rs4134779[c]		Probe-G	6FAM-**CCCTGCTCCGATTC**-MGB
		Primer-F	CAGTCAGTGGCCCAGAAGAC
		Primer-R	AGCATCAAGGATACCCCTCACA
TNFα(−308)[a]	G→A	Probe-G	VIC-**TGAGGGGCATGAGGACGGG**-TAMRA
rs1800629		Probe-A	6FAM-**AGGGGCATGGGGACGGG**-TAMRA
		Primer-F	CCCCAAAAGAAATGGAGGC
		Primer-R	TCTTCTGGGCCACTGACTGAT
IL-1β(−511)	C→T	Probe-C	VIC-**AGAGCTCCCGAGGC**-MGB
rs16944		Probe-T	6FAM-**AGCTCCTGAGGCAGA**-MGB
		Primer-F	CAGAGGCTCCTGCAATTGACA
		Primer-R	GGTCTCTACCTTGGGTGCTGTTC
IL-1β(+3953)	C→T	Probe-C	VIC-**TGTGTCGAAGAAGAT**-MGB
rs1143634		Probe-T	6FAM-**TCCCATGTGTCAAAGA**-MGB
		Primer-F	CCTAAACAACATGTGCTCCACATT
		Primer-R	ATCGTGCACATAAGCCTCGTTA
IL-1α(+4845)	G→T	Probe-G	VIC-**AGGTCAGCACCTTT**-MGB
rs17561		Probe-T	6FAM- **AGGTCATCACCTTTT**-MGB
		Primer-F	GCACTTGTGATCATGGTTTTAGAAA
		Primer-R	GTATTTCACATTGCTCAGGAAGCT
TGFβ1(−509)	C→T	Probe-C	VIC-**CCATCCCTCAGGTGT**-MGB
rs1800469		Probe-T	6FAM-**CATCCTTCAGGTGTC**-MGB
		Primer-F	AAGGAGAGCAATTCTTACAGGTGTCT
		Primer-R	GCCTCCGGAGGGTGTCA
IL-10(−819)	C→T	Probe-C	VIC-**AGGTGATGTAACATCT**-MGB
rs1800871		Probe-T	6FAM-**AGGTGATGTAATATCT**-MGB
		Primer-F	GAGGAAACCAAATTCTCAGTTAGCA
		Primer-R	TTATAGTGAGCAAACTGAGGCACAG
IL10(−1082)	A→G	Probe-A	VIC-**TCCCCTTCCCAAAGA**-MGB
rs1800896		Probe-G	6FAM-**TCCCCCTCCCAAAG**-MGB
		Primer-F	ACACACACAAATCCAAGACAACACT
		Primer-R	GCTGGATAGGAGGTCCCTTACTTT

[a] *TNFα*(−238) and *TNFα*(−308) SNP assays were obtained from the National Cancer Institute SNP500Cancer database under the auspices of the Cancer Genome Anatomy Project. The database is located at the following web address: http://snp500cancer.nci.nih.gov/home.cfm. All other assays were designed using Assay-by-Design™ service (Applied Biosystems).
[b] MGB, minor grove binder (Epoch Bioscience).
[c] National Center for Biotechnology Information (NCBI) dbSNP ID number.

different based on a two sided z-statistic ($z = 0.813$; $P = 0.4162$). Similar results have been obtained previously showing an error rate of <1 in 2000 for other polymorphisms genotyped by real-time PCR [15].

Another advantage of the real-time PCR assays used in the present study is ease of performance compared to PCR-RFLP. The entire test, including amplification and genotype identification is performed in a single closed tube (homogeneous). This homogeneity greatly reduces hands-on time and manipulations, which subsequently reduces the likelihood of cross contamination and genotyping miscalling. The reduction in hands-on time increases the throughput of real-time PCR genotyping and also reduces the cost of performing the assays. The throughput for our lab has increased from approximately 6–24 h/80 genotypes by PCR-RFLP to

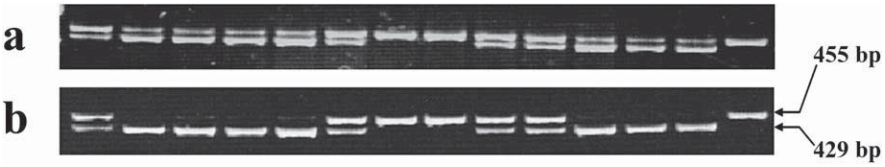

Fig. 2. Genotyping results for *TGFβ1*(−509) SNP using PCR-RFLP. Conditions for RFLP are given in Table 3. The CC genotype (major variant) has a cleavage site for *Eco 81 I* and results in two bands at 429 bp (lower band in figure) and 26 bp (not shown). The C to T transition eliminates this cleavage site. Therefore the CT genotype give three bands (455, 429 and 26 bp) and the TT genotype gives one band at 455 bp indicating no enzyme cleavage. Results for the same samples digested with 10 U (a) and 20 U (b) are shown demonstrating the possibility of misidentification of genotypes due to under digestion of the PCR product.

less than 2 h/80 genotypes by real-time PCR assay with only 20 min hands-on-time. In addition, PCR-RFLP demands manual calling of the genotypes in a blinded manner by two independent readers. The automatic genotype calling performed by the IQ software, one of several commercial programs available, increases the speed with which the genotypes are attained and helps eliminate one source of human error in the identification process. Similar automatic genotype identification can be performed using a Microsoft Excel macro when using a regular thermal cycler and a fluorescent plate reader. Statistical clustering methods are also available [15,16]. These advantages should contribute to an overall reduction in cost as well as error rate. The reagent cost for the RFLP assays used in the present study is estimated at $3.50/sample and for real-time PCR assays is estimated at $0.85/sample. This indicates that real-time PCR genotyping can reduce costs up to 75% over traditional RFLP methods. Throughput can easily be increased beyond that stated in this publication by automating the setup of the assays and also switching to a 384-well or greater format thus further decreasing the time and cost associated with genotyping large populations.

Organized databases available to the scientific community will greatly facilitate research investigating the association of cytokine polymorphisms with human disease. *Genes and Immunity* manages an extensive on-line public database (http://bris.ac.uk/pathandmicro/services/GAI/cytokine4.htm) focused on cytokine polymorphisms and their impact on in vitro cytokine expression and in vivo disease associations [17]. A new public database managed by the National Cancer Institute is being established and is termed the SNP500Cancer database (http://snp500cancer.nci.nih.gov/home.cfm). This database contains validated (by direct sequencing) SNP genotyping assays including Taqman®, Epoch MGB Eclipse™ and Sequenom®. New assays are continuously being added to the database as they are validated. A manuscript describing the utility of information on the site and the validation process for the assays is being prepared by the SNP500Cancer group at the National Cancer Institute (Drs. Robert Welch and Stephen Chanock, personal communication). These two databases are enormous assets to disease genomics research and will facilitate interlaboratory comparison and validation of real-time PCR genotyping through the use of common assays. All of the real-time PCR genotyping assays in the present study have been validated by direct sequencing (by NCI) in addition to our validation by PCR-RFLP and can be found in the SNP500Cancer database.

In summary, this study describes real-time PCR allelic discrimination assays for eight functionally important cytokine polymorphisms. These assays can be implemented in large population studies examining the association between cytokine SNPs and complex disease. Our laboratory is currently using these assays to examine the association of cytokine genetic variation in occupational disease prevalence and severity. It is evident that the real-time PCR technique overcomes many of the difficulties of traditional PCR-RFLP and greatly increases the throughput capabilities of genotyping. In addition to Taqman® assays, the MGB Eclipse™ hybridization probe assays (Epoch Bioscience) are an alternative real-time PCR allelic discrimination technique with similar advantages over traditional PCR-RFLP and are being validated in the SNP500Cancer database.

3. Materials and methods

3.1. Real-time PCR SNP Genotyping

Real-time PCR allelic discrimination assays were designed using Primer Express® software and the Assay-by-Design℠ service offered by Applied Biosystems (Foster City, CA). Primer and probe combinations designed using Assay-by-Design℠ service are listed in Table 3. All probe/primer sets were designed to function using universal reaction and cycling conditions. Genotyping was performed in 25 μl reactions consisting of 10 ng of genomic DNA, 900 nM of each primer, 200 nM of each probe and 12.5 μl of 2× Taqman® Universal PCR Master Mix (contains PCR buffer, passive reference dye ROX, deoxynucleotides, uridine, uracil-*N*-glycosylase and AmpliTaq Gold® DNA polymerase; Perkin-Elmer, Applied Biosystems, Foster City, CA). Amplification was performed using an iCycler® IQ (Bio-Rad Laboratories, Hercules, CA) real-time thermal cycler. Cycling conditions were 2 min at 50 °C; 10 min at 95 °C; 50 cycles of 92 °C for 30 s; 60 °C for 1 min. Real-time fluorescence detection was performed during the 60 °C annealing/extension step of each cycle. The IQ software (Bio-Rad Laboratories, Hercules, CA) was used to plot and automatically call genotypes based on a two parameter plot using fluorescence intensities of FAM and VIC at 49 cycles. This software uses auto-scaling for the allelic discrimination plot. Therefore, caution must be taken when analyzing data for cytokine SNPs with rare minor alleles. If no minor alleles are present in the run, the software will miscall genotypes as heterozygous. To prevent this, it is essential that a positive control for both alleles and a heterozygous sample be included with each genotyping plate.

In separate experiments, PCR reactions were performed using the same DNA samples as well as reaction and cycling conditions in a 96-well GeneAmp® 9600 thermal cycler (Perkin-Elmer, Applied Biosystems, Foster City, CA). Wells from these reactions were read using an ABI 7700 (real-time PCR instrument) and/or

Table 3
Cytokine polymorphism PCR-RFLP primer combinations, cycling parameters and restriction patterns

Cytokine SNP	Reagent	Sequence 5′ → 3′/endonuclease	Thermal cycling	Restriction pattern (bp)
TNFα(−238) [18]	Primer-F	GAAGCCCCTCCCAGTTCTAGTTC	94 °C, 3 min	AA 63+49+21
rs4134779[a]	Primer-R	CACTCCCCATCCTCCCTGGTC	94 °C, 1 min; 61 °C, 1 min; 72 °C, 1 min **X 35** cycle	AG 70+63+49+21
	Enzyme[b]	5 U *Ava* II; 37 °C, for 2 h	72 °C, 5 min	GG 70+63
TNFα(−308) [18]	Primer-F	AGGCAATAGGTTTTGAGGGCCAT	95 °C, 1 min	GG 87+20
rs1800629	Primer-R	TCCTCCCTGCTCCGATTCCG	94 °C, 1 min; 60 °C, 1 min; 72 °C, 1 min **X 35** cycle	GA 107+87+20
	Enzyme	6 U *Nco* I; 37 °C, for 2 h	72 °C, 5 min	AA 107
IL-1β(−511) [18]	Primer-F	TGGCATTGATCTGGTTCATC	95 °C, 1 min	CC 190+114
rs16944	Primer-R	GTTTAGGAATCTTCCCACTT	95 °C 1 min, 53 °C, 1 min, 72 °C, 1 min **X 35** cycle	CT 304+190+114
	Enzyme	3 U *Ava* I; 37 °C, for 2 h	72 °C, 5 min	TT 304
IL-1β(+3953) [18]	Primer-F	CTCAGGTGTCCTCGAAGAAATCAAA	95 °C, 2 min	CC 97+85
rs1143634	Primer-R	GCTTTTTTGCTGTGAGTCCCG	95 °C, 1 min, 67.5 °C, 1 min, 72 °C, 1 min **X 35** cycle	CT 182+97+85
	Enzyme	10 U *Taq* I; 65 °C, for 2 h	72 °C, 5 min	TT 182
IL-1α(+4845) [18]	Primer-F	ATGGTTTTAGAAATCATCAAGCCTAGGGCA	95 °C, 1 min	GG 124+76+29
rs17561	Primer-R	AATGAAAGGAGGGGAGGATGACAGAAATGT	95 °C, 1 min; 56 °C, 1 min; 72 °C, 2 min **X 35** cycle	GT 153+124+76+29
	Enzyme	2.5 U *Fm*u4H1; 37 °C, for 2 h	72 °C, 5 min	TT 153+76
TGFβ1(−509) [19]	Primer-F	GGGGACACCATCTACAGTG	94 °C, 5 min	CC 429+26
rs1800469	Primer-R	GGAGGAGGGGGCAACAGG	94 °C, 30 s; 60 °C, 30 s; 72 °C, 30 s **X 35** cycle	CT 455+429+26
	Enzyme	20 U *Eco* 81 I; 37 °C, for 18 h	72 °C, 5 min	TT 455
IL-10(−819) [20]	Primer-F	ATCCAAGACAACACTACTAA	95 °C, 3 min	CC 292+217+79
rs1800871	Primer-R	TAAATATCCTCAAAGTTCC	95 °C, 30 s; 56 °C, 30 s; 72 °C, 1 min **X 30** cycle	CT 509+292+217+79
	Enzyme	10 U *Mae* III; 55 °C, for 2 h	72 °C, 3 min	TT 509+79
IL10(−1082) [21]	Primer-F	CCAAGACAACACTACTAAGGCTTCTTGAGGA	95 °C, 10 min	AA 360
rs1800896	Primer-R	AGGTAGTGCTCACCATGACC	95 °C, 30 s; 58 °C, 30 s; 72 °C, 45 s **X 35** cycle	AG 360+320
	Enzyme	10 U *Bse* RI; 37 °C, for 18 h	72 °C, 10 min	GG 320

[a] National Center for Biotechnology Information (NCBI) dbSNP ID.
[b] Restriction endonuclease used to digest respective PCR products.

an LS 50 luminescence spectrometer at 490/520 nm excitation/emission (FAM) and 530/560 nm excitation/emission (VIC). The fluorescence data were plotted using Microsoft Excel and genotypes were called automatically using Genotyper-Endpoint, an Excel macro created by Max Myakishev (NCI, Bethesda, MD). Genotypes were identified in 100% concordance with the automatic genotyping assignments performed by the IQ software following real-time PCR on the iCycler instrument (data not shown). In order to automate the identification of alleles using a normal thermal cycler and fluorescent plate reader, we recommend using Genotyper-Endpoint or K-means clustering statistical analysis previously published by Olivier et al. [16] and Ranade et al. [17].

3.2. PCR-RFLP SNP genotyping

Genotyping was performed using PCR-RFLP techniques as previously described [18]. Briefly, 20 ng/50 μl genomic DNA was placed in 10 mM Tris–HCl, 50 mM KCl buffer containing 0.2 mM of each dNTP, 1.25 units of *Taq* polymerase with primer sets specific for *TNFα* (−238), *TNFα*(−308), *IL-1β*(−511), *IL-1β*(+3953), *IL-1α*(+4845), *TGFβ1*(−509), *IL-10*(−819) and *IL-10* (−1082) SNP sites. Primer sets, enzymes, thermal cycling conditions and restriction patterns are summarized in Table 1. Following amplification, 20 μl of reaction was digested using the respective enzyme (Table 1). The products were separated on 10% polyacrylamide-TBE gels (Bio-Rad Laboratories, Hercules, CA) and visualized by ethidium bromide/UV illumination. Manual genotype calling was performed in a blinded manner by two independent readers according to the digestion patterns specific for each cytokine SNP (Table 1).

Acknowledgements

The authors would like to thank Dr. Michael Kashon, Nancy Bollinger and Dr. Channa Keshava for their critical review of this manuscript during its preparation. All experimentation complies with the mandates of the NIOSH Institutional Review Board and the current laws of the United States of America.

References

[1] Shastry BS. SNP alleles in human disease and evolution. J Hum Genet 2002;47:561–6.
[2] Brookes AJ. The essence of SNPs. Gene 1999;234:177–86.
[3] McCarthy JJ, Hilfiker R. The use of single-nucleotide polymorphism maps in pharmacogenomics. Nat Biotechnol 2000;18:505–8.
[4] Riley JH, Allan CJ, Lai E, Roses A. The use of single nucleotide polymorphisms in the isolation of common disease genes. Pharmacogenomics 2000;1:39–47.
[5] Lai E. Application of SNP technologies in medicine: lessons learned and future challenges. Genome Res 2001;11:927–9.
[6] Tabor HK, Risch NJ, Myers RM. Opinion: Candidate-gene approaches for studying complex genetic traits: practical considerations. Nat Rev Genet 2002;3:391–7.
[7] Risch N, Merikangas K. The future of genetic studies of complex human diseases. Science 1996;273:1516–7.
[8] Lazarus R, Vercelli D, Palmer LJ, Klimecki WJ, Silverman EK, Richter B, et al. Single nucleotide polymorphisms in innate immunity genes: abundant variation and potential role in complex human disease. Immunol Rev 2002;190:9–25.
[9] Palmer LJ, Cookson WO. Using single nucleotide polymorphisms as a means to understanding the pathophysiology of asthma. Respir Res 2001;2:102–12.
[10] Shi MM. Enabling large-scale pharmacogenetic studies by high-throughput mutation detection and genotyping technologies. Clin Chem 2001;47:164–72.
[11] Landegren U, Nilsson M, Kwok PY. Reading bits of genetic information: methods for single-nucleotide polymorphism analysis. Genome Res 1998;8:769–76.
[12] Cheng S, Grow MA, Pallaud C, Klitz W, Erlich HA, Visvikis S, et al. A multilocus genotyping assay for candidate markers of cardiovascular disease risk. Genome Res. 1999;9:936–49.
[13] Yucesoy B, Kashon ML, Luster MI. Cytokine polymorphisms in chronic inflammatory diseases with reference to occupational diseases. Curr Mol Med 2003;3:39–48.
[14] Haring D, Endler G, Hsieh K, Mannhalter C. Comparison of methods for determination of interleukin 6 G(−174)C promoter polymorphism. Biotechniques 2002;33:1114–7.
[15] Ranade K, Chang MS, Ting CT, Pei D, Hsiao CF, Olivier M, et al. High-throughput genotyping with single nucleotide polymorphisms. Genome Res 2001;11:1262–8.
[16] Olivier M, Chuang LM, Chang MS, Chen YT, Pei D, Ranade K, et al. High-throughput genotyping of single nucleotide polymorphisms using new biplex invader technology. Nucleic Acids Res 2002;30:e53.
[17] Haukim N, Bidwell JL, Smith AJ, Keen LJ, Gallagher G, Kimberly R, et al. Cytokine gene polymorphism in human disease: on-line databases. Genes Immun 2002;3(Suppl 2):313–30.
[18] Yucesoy B, Vallyathan V, Landsittel DP, Sharp DS, Weston A, Burleson GR, et al. Association of tumor necrosis factor-alpha and interleukin-1 gene polymorphisms with silicosis. Toxicol Appl Pharmacol 2001;172:75–82.
[19] Lario S, Inigo P, Campistol JM, Poch E, Rivera F. Oppenheimer F Restriction enzyme-based method for transforming growth factor-beta(1) genotyping: nonisotopic detection of polymorphisms in codons 10 and 25 and the 5′-flanking region. Clin Chem 1999;45:1290–2.
[20] Mok CC, Lanchbury JS, Chan DW, Lau CS. Interleukin-10 promoter polymorphisms in Southern Chinese patients with systemic lupus erythematosus. Arthritis Rheum 1998;41:1090–5.
[21] De Jong BA, Westendorp RG, Eskdale J, Uitdehaag BM, Huizinga TW. Frequency of functional interleukin-10 promoter polymorphism is different between relapse-onset and primary progressive multiple sclerosis. Hum Immunol 2002;63:281–5.