Benchmark

Comparison of Methods for Determination of Interleukin 6 G(-174)C Promoter Polymorphism

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Interleukin 6 (IL-6) is one of the key proinflammatory cytokines that is produced by many different cell types, including cells of the immune system, endothelial cells, fibroblasts, myocytes, and adipocytes. It can mediate inflammatory but also stress-induced responses (10,14–16). IL-6 induces the secretion of monocyte chemotactic protein, an important mediator of inflammatory events in atherosclerosis, and regulates the expression of adhesion molecules and the release of cytokines such as TNF- α and IL-1 β (3,13). IL-6 basal and peak levels show inter-individual variability determined in part by genetic factors (4,12). Recently, a G-to-C polymorphism at nucleotide position (-174) in the promoter region of the IL-6 gene has been identified by Fishman et al. (9) and has been associated with an altered transcriptional response to stimuli such as endotoxin or IL-1 (8). In some studies, individuals carrying the GG genotype have been reported to show higher plasma levels for IL-6 in comparison to carriers of the CC genotype (5,8). However, controversial data in individuals homozygous for the CC genotype have also been described (1). This polymorphism has been associat-

ed with systemic-onset juvenile chronic arthritis (8), osteoporosis (8), atherosclerosis (17), the incidence and outcome of sepsis (19), susceptibility to type 1 diabetes mellitus (11), coronary heart disease (1), or IL-6 levels after coronary artery bypass surgery (4).

To clarify, if and how the polymorphism in the promoter region of the IL-6 gene influences plasma levels and the predisposition to various common diseases, studies on large cohorts of patients and control individuals are required. Thus, fast, reliable, and economical assays for determination of the IL-6 promoter polymorphism have been developed and described in the literature. However, up to now no direct comparison of the different assays in regard to reliability and economic aspects has yet been performed.

We compared different methods, PCR followed by restriction enzyme digestion, a newly developed mutagenically separated PCR, and real-time PCR using fluorescence-labeled hybridization probes, for their reliability, the required working time, and reagent costs.

For all tests, DNA from 50 individuals was extracted from venous blood anticoagulated with EDTA using the MagNA Pure LC isolation system (Roche Applied Science, Mannheim Germany). All individuals had given their informed consent to DNA analysis.

PCR amplification for restriction enzyme digestion was carried out following a published protocol (7) with primers forward 5'-TGACTTCAGCTTTACTCTTTGT-3' and reverse 5'-CT-

GATTGGAAACCTTATTAAG-3' (Tib Molbiol, Berlin, Germany). PCR was performed in an Eppendorf® cyclor in a final volume of 50 μ L containing approximately 50 ng DNA, 1.5 mM MgCl₂, 200 μ M each dNTP (Amersham Bioscience, Piscataway, NJ, USA), 10 pmol each primer, and 1.25 U AmpliTaq Gold® (Applied Biosystems, Foster City, CA, USA). DNA was amplified for 35 cycles of denaturation for 40 s at 95°C, annealing for 40 s at 55°C, and elongation for 40 s at 72°C. A final extension for 5 min at 72°C completed the reaction. PCR amplification was followed by restriction digestion with the enzyme *Sfa*NI (New England Biolabs, Hertfordshire, UK) at 37°C for 3 h. The digested products were electrophoretically separated on 6% precast polyacrylamide Novex® gels (Invitrogen, Carlsbad, CA, USA) for 50 min at 160 V. Gels were stained with SYBR Green® (1:10 000; Molecular Probes, Eugene, OR, USA). Individuals carrying the GG genotype showed bands at 140 and 58 bp, individuals with the heterozygous GC genotype had bands at 198, 140, and 58 bp, and individuals homozygous for the CC genotype had one band at 198 bp.

The new allele-specific PCR method developed by us is based on the principle of mutagenically separated PCR (18), a single-tube PCR technique with allele-specific primers differing in length by 9 bp. Base mismatches in the allele-specific primers introduce differences into the PCR products that minimize cross-reactions of the products in

subsequent cycles. Because of the 9-bp length difference, the alleles are easily discernible by high-resolution electrophoresis. Mutagenically separated PCRs were performed in a GeneAmp® PCR System 9700 (Applied Biosystems) using 50-µL reaction volumes containing 5 µL 10× buffer, approximately 50 ng DNA, 1.5 mM MgCl₂, 200 µM each dNTP, 1.25 U AmpliTaq Gold, and the following primers: 13 pmol IL-6 (-174G) forward primer (5'-TCCCCCTAGTTGTGTCTCGCG-3'), 5 pmol IL-6 (-174C) forward primer (5'-CTGCACTTTATCCCCCTAGTTGTGTCATGCC-3'), and 13 pmol IL-6 common reverse primer (5'-TGAGGTGGGGCCAGAGC-3') (all from Tib Molbiol). Mismatches with wild-type sequences are underlined. A 10-min denaturation at 95°C was followed by 37 cycles at 95°C for 30 s, 58°C for 45 s, and 72°C for 60 s, and a final extension step of 7 min at 72°C. The PCR products were analyzed by gel electrophoresis on 6% precast polyacrylamide Novex gels at 160 V for 50 min and stained for 20 min with SYBR Green (1:10 000 dilution). The PCR product generated from the G-allele has a length of 95 bp; the C-allele gives a 104-bp product.

All 50 individuals were also tested with a real-time PCR assay following a published protocol. Primers and fluorescent hybridization probes (2) were used in modified concentrations. PCR was performed in a reaction volume of 20 µL containing approximately 50 ng DNA, 10 pmol forward primer 5'-TTACTCTTTGTCAAGACATGCCA-3' and reverse primer 5'-ATGAGCCTCAGACATCTCCAG-3' (Tib Molbiol), 2 µL reaction buffer (Roche Applied Science), 2.25 mM MgCl₂, and 2 pmol each of the labeled probes. The anchor probe (5'-CTAAGCTGCACTTTTCCCCCTAGT-3') was labeled at the 3'-end with fluorescein; the sensor probe, specific for the G allele (5'-GTGTCTTGCGATGCTAAAGGA-3'), was labeled with LightCycler™ Red 640.

An initial denaturation at 95°C for 10 min was followed by 45 cycles of denaturation at 95°C for 10 s (at a rate of 20°C/s), annealing at 53°C for 10 s, and extension at 72°C for 10 s. The melting curve started at 95°C, followed by 45°C for 90 s. Then, the temperature was in-

creased to 85°C at a rate of 0.1°C/s.

Table 1 shows the genotype results obtained with the three methods for the 50 samples, the reagent costs, and the working time per sample.

For three individuals, we observed discrepant results by RFLP analysis, mutagenically separated PCR, and real-time PCR. By RFLP assay, the three samples were heterozygous, whereas they were homozygous GG by mutagenically separated PCR and real-time PCR. After direct sequencing using the Genetic Analyzer ABI Prism® System 310 (Applied Biosystems) with four-color fluorescence and the primers described for RFLP analysis, the genotype of the three samples was confirmed to be GG.

It is highly likely that the apparent heterozygous genotype pattern was due to incomplete digestion. The PCR fragment generated by the published primers does not contain a mutation-independent internal cleavage site for *Sfa*NI that would allow one to control for incomplete digestion. Even though PCR amplifications followed by restriction enzyme digestion are simple and robust methods that have been used for the detection of numerous sequence variations, one must realize that assays that do not include a mutation-independent restriction site can give unreliable results because of incomplete digestion. When developing new RFLP-based mutation detection assays, mutation-independent cleavage sites should be introduced within the PCR product. As can be seen from our working time and cost calculations, RFLP analyses often require the use of relatively expensive restriction enzymes, for the IL-6 polymorphism *Sfa*NI, and additional pipetting steps that also contribute to the increase of bench working time and costs.

Real-time PCR represents a fast and elegant method that can be completely automated (e.g., by combining the MagNA Pure pipetting unit with the LightCycler PCR machine). Interestingly, the bench working time per sample was similar for real-time and mutagenically separated PCR. This was mainly because a maximum of 32 samples can be analyzed simultaneously in the LightCycler, while 96 samples can be tested in the GeneAmp PCR System 9700. The application of an aliquot of

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the PCR product onto the precast Novex gels by an experienced person takes less than 5 s/sample, and the total electrophoresis working time is 70 min for 96 samples.

Reagents costs per sample were relatively high for real-time PCR. While the limit of 32 samples in the LightCycler can be overcome when using the Taq-Man® (Applied Biosystems) or equivalent other real-time PCR systems, expensive laboratory equipment and reagents are also required for these systems.

In contrast, the allele-specific mutagenically separated PCR is a cheap, fast, simple, and robust method that allows mutation detection using standard PCR reagents and equipment. Importantly, the mutagenically separated PCR is performed as self-controlling single-tube assay that allows reliable detection of genotyping mistakes due to inefficient amplification, insufficient DNA quantity or quality, or pipetting mis-

Table 1. Genotype Results, Active Working Time, and Costs per Sample

Method	PCR and Restriction Enzyme Digestion	Mutagenically Separated PCR	LightCycler Real-Time PCR	Sequencing ^a
Genotype	21 GG (42)	24 GG (48)	24 GG (48)	
Results	23 GC (46)	20 GC (40)	20 GC (40)	
n (% of total)	6 CC (12)	6 CC (12)	6 CC (12)	
Working Time/Sample	8 min	4 min	4 min	35 min
Total Time Required for 50 Samples	12 h	7 h	7.5 h	11.5 h
Reagent Costs/Sample (US dollars)	\$12	\$2.50	\$11	\$73

^aPlease note that only five samples were analyzed by direct sequencing.

takes, as at least one PCR product has to be amplified in each tube. Recently, another mutagenically separated PCR assay for analysis of the IL-6 G(-174)C

polymorphism has been successfully employed (19), confirming the reliability and stability of this method. For mutation detection, several other

techniques have been proposed to avoid gel electrophoresis such as allele-specific hybridization onto solid-phase bound oligonucleotides (6). These techniques allow simultaneous analysis of numerous polymorphisms in a multiplexed fashion (e.g., on microarrays). However, all hybridization techniques for detection of SNPs carry the danger of non-specific hybridization and unreliable results. Thus, they have to be carefully validated and standardized before applying them to daily routine. Although these methods can be partly automated, they require high efforts in working time and are still quite costly. In conclusion, the mutagenically separated PCR described here is a fast (4 min/individual test), reproducible, and cheap single-tube reaction for which no special equipment is needed. However, the method can be adapted for use in an automated PCR system. With this technique, the total actual working time can be cut in half compared to PCR amplification with subsequent enzyme digestion. Compared to real-time PCR, costs are clearly lower. The outstanding feature of the mutagenically separated PCR is the performance as self-controlling single-tube assay that allows reliable detection of genotyping mistakes and makes the digestion procedure unnecessary. Even though this multiplex PCR is very stable and reliable, we recommend that users reevaluate the PCR conditions and the primer concentration for their laboratory equipment and reagents.

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REFERENCES

1. Basso, F., G.D. Lowe, A. Rumley, A.D. McMahon, and S.E. Humphries. 2002. Interleukin-6 -174G>C polymorphism and risk of coronary heart disease in West of Scotland coronary prevention study (WOSCOPS). *Arterioscler. Thromb. Vasc. Biol.* 22:599-604.
2. Bertsch, T., W. Zimmer, W. Casarin, C. Denz, M. Quintel, and K. Fassbender. 2001. Real-time PCR assay with fluorescent hybridization probes for rapid interleukin-6 promoter (-174G→C) genotyping. *Clin. Chem.* 47:1873-1874.
3. Biswas, P., F. Delfanti, S. Bernasconi, M. Mengozzi, M. Cota, N. Polentarutti, A. Mantovani, A. Lazzarin, et al. 1998. Interleukin-6 induces monocyte chemotactic protein-1 in peripheral blood mononuclear cells and in the U937 cell line. *Blood* 91:258-265.
4. Brull, D.J., H.E. Montgomery, J. Sanders, S. Dhamrait, L. Luong, A. Rumley, G.D. Lowe, and S.E. Humphries. 2001. Interleukin-6 gene -174g>c and -572g>c promoter polymorphisms are strong predictors of plasma interleukin-6 levels after coronary artery bypass surgery. *Arterioscler. Thromb. Vasc. Biol.* 21:1458-1463.
5. Burzotta, F., L. Iacoviello, A. Di Castelnuovo, F. Glicca, N. Luciani, R. Zamparelli, R. Schiavella, M.B. Donati, et al. 2001. Relation of the -174 G/C polymorphism of interleukin-6 to interleukin-6 plasma levels and to length of hospitalization after surgical coronary revascularization. *Am. J. Cardiol.* 88:1125-1128.
6. Cheng, S., M.A. Grow, C. Pallaud, W. Klitz, H.A. Erlich, S. Visvikis, J.J. Chen, C.R. Pullinger, et al. 1999. A multilocus genotyping assay for candidate markers of cardiovascular disease risk. *Genome Res.* 9:936-949.
7. Fernandez-Real, J.M., M. Broch, J. Vendrell, C. Richart, and W. Ricart. 2000. Interleukin-6 gene polymorphism and lipid abnormalities in healthy subjects. *J. Clin. Endocrinol. Metab.* 85:1334-1339.
8. Ferrari, S.L., P. Garnero, S. Emond, H. Montgomery, S.E. Humphries, and S.L. Greenspan. 2001. A functional polymorphic variant in the interleukin-6 gene promoter associated with low bone resorption in postmenopausal women. *Arthritis Rheum.* 44:196-201.
9. Fishman, D., G. Faulds, R. Jeffery, V. Mohamed-Ali, J.S. Yudkin, S. Humphries, and P. Woo. 1998. The effect of novel polymorphisms in the interleukin-6 (IL-6) gene on IL-6 transcription and plasma IL-6 levels, and an association with systemic-onset juvenile chronic arthritis. *J. Clin. Invest.* 102:1369-1376.
10. Fried, S.K., D.A. Bunkin, and A.S. Greenberg. 1998. Omental and subcutaneous adipose tissues of obese subjects release interleukin-6: depot difference and regulation by glucocorticoid. *J. Clin. Endocrinol. Metab.* 83:847-850.
11. Jahromi, M.M., B.A. Millward, and A.G. Demaine. 2000. A polymorphism in the promoter region of the gene for interleukin-6 is associated with susceptibility to type 1 diabetes mellitus. *J. Interferon Cytokine Res.* 20:885-888.
12. Kilpinen, S., J. Hukkonen, X.Y. Wang, and M. Hurme. 2001. The promoter polymorphism of the interleukin-6 gene regulates interleukin-6 production in neonates but not in adults. *Eur. Cytokine Netw.* 12:62-68.
13. Mantovani, A. 1997. The interplay between primary and secondary cytokines. Cytokines involved in the regulation of monocyte recruitment. *Drugs* 54(Suppl1):15-23.
14. Mohamed-Ali, V., S. Goodrick, A. Rawesh, D.R. Katz, J.M. Miles, J.S. Yudkin, S. Klein, and S.W. Coppack. 1997. Subcutaneous adipose tissue releases interleukin-6, but not tumor necrosis factor-alpha, in vivo. *J. Clin. Endocrinol. Metab.* 82:4196-4200.
15. Orban, Z., A.T. Remaley, M. Sampson, Z. Trajanoski, and G.P. Chrousos. 1999. The differential effect of food intake and β -adrenergic stimulation on adipose-derived hormones and cytokines in man. *J. Clin. Endocrinol. Metab.* 84:2126-2133.
16. Papanicolaou, D.A., R.L. Wilder, S.C. Manolagas, and G.P. Chrousos. 1998. The pathophysiologic roles of interleukin-6 in human disease. *Ann. Intern. Med.* 128:127-137.
17. Rauramaa, R., S.B. Vaisanen, L.A. Luong, A. Schmidt-Trucksass, I.M. Penttila, C. Bouchard, J. Toyry, and S.E. Humphries. 2000. Stromelysin-1 and interleukin-6 gene promoter polymorphisms are determinants of asymptomatic carotid artery atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* 20:2657-2662.
18. Rust, S., H. Funke, and G. Assmann. 1993. Mutagenically separated PCR (MS-PCR): a highly specific one step procedure for easy mutation detection. *Nucleic Acids Res.* 21:3623-3629.
19. Schlüter, B., C. Raufhake, M. Erren, H. Schotte, F. Kipp, S. Rust, A.H. Van Aken, G. Assmann, and E. Berendes. 2002. Effect of the interleukin-6 promoter polymorphism (-174 G/C) on the incidence and outcome of sepsis. *Crit. Care Med.* 30:32-37.

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