Restriction Enzyme-based Method for Transforming Growth Factor-β1 Genotyping: Nonisotopic Detection of Polymorphisms in Codons 10 and 25 and the 5′-Flanking Region, Sergio Lario,1 Pablo In˜igo,1 Josep M. Campistol,1* 

we refer to the 5′-flanking region of the TGFβ1 gene (at positions −988, −800, and −509), three were in the coding region (codons 10, 25, and 263), and an insertion was also described in the 5′-untranslated region at position +72.

The analysis of candidate genes and/or polymorphisms has been widely applied in clinical genetics to study human diseases. A rapid and easy method for genotyping is essential in such studies, in which large numbers of cases and controls are needed. The ECTIM study used allele-specific oligonucleotides (ASOs). After amplification by PCR, products were blotted into a nylon membrane and hybridized with an ASO probe. The major disadvantage of this method is that it its time-consuming and requires radioactively labeled oligonucleotides.

We describe here a new, rapid, nonradioactive, and relatively inexpensive method for TGFβ1 genotyping, using restriction site polymorphism (RSP) methodology. A fragment of the promoter or coding region of the TGFβ1 gene is amplified by PCR and subsequently digested with restriction enzymes: MaeIII, AciI, MspAI, and FseI for the allelic variants located at positions −800, −509, codon 10, and codon 25, respectively.

We studied DNA from 73 cadaver organ donors from our Institution with no associated medical disease (e.g., arterial hypertension, any type of nephropathy, chronic obstructive pulmonary diseases, and chronic liver diseases) and no chronic diseases. Blood samples (10 mL) were collected into EDTA tubes and used for the separation of white cells. Genomic DNA was purified from peripheral leukocytes by salting out (4).

Published sequence information of the TGF-β1 promoter gene (2) was used to design a pair of primers spanning positions −800 and −590; this amplification will be referred to as F1-F2. Primers for codons 10 and 25 were kindly provided by I. Hutchinson (University of Manchester, UK), and this amplification will be referred to as F3-F4. The primer positions, sequences, and product sizes for the F1-F2 and F3-F4 amplifications are listed in Table 1, and a schematic showing the positions of both polymorphic and nonpolymorphic restriction sites is shown in Fig. 1A.

PCR conditions were the same for both amplifications: 1.5 mmol/L MgCl₂, 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 1 μmol/L each primer, 0.2 mmol/L each dNTP, 25 ng of genomic DNA, 2.0 U of Taq DNA polymerase (Boehringer Mannheim), and 50 mL/L formamide in a 30-μL reaction mixture. PCR contamination was checked by the inclusion of negative controls. PCR reactions were initially denatured at 94 °C for 5 min and subjected to 35 cycles in a PTC-100 thermal cycler (MJ Research) with 30 s of denaturing at 94 °C, 30 s of annealing at 60 °C, and 30 s of extension at 72 °C. A final elongation step of 5 min at 72 °C was also included. The identities of the F1-F2 and F3-F4 amplification products were confirmed by restriction enzyme digestion (data not shown). Amplified DNA (5–10 μL) was digested in a 20-μL reaction mixture, using the buffers and temperatures recommended by the manufacturers. The enzymes used were as follows: MaeIII and AciI (Boehringer Mannheim),
MspI and FseI (New England Biolabs). Digestions were carried out for at least 2 h. The amount of restriction enzyme required for each digestion was as follows: 1.5 U for MaeIII, 5.0 U for AocI, 10.0 U for MspIAI, and 2.0 U for FseI. Digested products were fractionated in 7.0% polyacrylamide gels and visualized by ethidium bromide staining and transillumination with ultraviolet light. The size marker was pBR322 cleaved with HaeIII (DNA molecular size marker V; Boehringer Mannheim).

Hardy-Weinberg equilibrium was tested by \( \chi^2 \) with one degree of freedom. Comparisons of genotype frequencies between the ECTIM control group and the present results were performed by \( \chi^2 \) with two degrees of freedom. In cases involving RSPs with low frequencies, groups were combined and subjected to the Fisher exact test.

Results for the AocI and MaeIII digestion for the \( 5' \)-end RSPs of the \( \text{TGFB1} \) gene are shown in Fig. 1B. The G\( \rightarrow \)A transition at position 2800 was detected by the loss of the restriction site for MaeIII: the G allele is defined by the presence of two polymorphic bands of 205 and 137 bp, whereas the A allele is defined by the absence of the MaeIII site and is detected by a 342-bp band.

Fig. 1. \( \text{TGFB1} \) genotyping by restriction enzyme digestion. (A), schematic showing the position of both polymorphic and nonpolymorphic restriction sites. (B), band patterns and genotype frequencies for −509 RSP (AocI digestion), −800 RSP (MaeIII digestion), codon 10 (MspA1I), and codon 25 (FseI) polymorphisms.

<table>
<thead>
<tr>
<th>Amplification</th>
<th>Primer sequence 5’ to 3’</th>
<th>Primer length, nt(^a)</th>
<th>5’ end position(^b)</th>
<th>Product length, bp</th>
<th>RSP</th>
<th>Enzyme</th>
<th>Recognition sequence,(^c) 5’ to 3’</th>
<th>Base substitution</th>
<th>Restriction site</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1–F2</td>
<td>F1(S) GGGGACACCATCAGTG</td>
<td>19</td>
<td>−937</td>
<td>455</td>
<td>−800</td>
<td>MaeII</td>
<td>G(\rightarrow)A</td>
<td>G(\rightarrow)A</td>
<td>Present</td>
</tr>
<tr>
<td>F2(As)</td>
<td>GGAGGGGAGGGGCAACAGG</td>
<td>18</td>
<td>−500</td>
<td>−509</td>
<td>AocI</td>
<td>CC(\rightarrow)TNAGG</td>
<td>C(\rightarrow)T</td>
<td>Present</td>
<td></td>
</tr>
<tr>
<td>F3–F4</td>
<td>F3(S) TCGCGGCTTTGTTGACA</td>
<td>20</td>
<td>+597</td>
<td>500</td>
<td>Codon 10</td>
<td>MspA1I</td>
<td>CTG(\rightarrow)CTG(^d)</td>
<td>T(\rightarrow)C (Leu(\rightarrow)Pro)</td>
<td>Absent</td>
</tr>
<tr>
<td>F4(As)</td>
<td>TCGCGGCTTTGTTGACA</td>
<td>19</td>
<td>+1078</td>
<td>455</td>
<td>Codon 25</td>
<td>FseI</td>
<td>GGCCGG(\rightarrow)CC</td>
<td>G(\rightarrow)C (Arg(\rightarrow)Pro)</td>
<td>Present</td>
</tr>
</tbody>
</table>

\(^a\) nt, nucleotides; S, sense; As, antisense.

\(^b\) Nucleotides are numbered from the start of transcription.

\(^c\) Underlined letters indicate the allelic substitutions determining the absence or presence of the restriction site.

\(^d\) The published sequence surrounding codon 10 is shown. The actual MspA1I restriction site is C(A or C)G\(\rightarrow\)C(G or T)G.
in the present studies; the differences are not statistically significant.

The major advantage of this method is its simplicity because only two PCR reactions are used to genotype four RSFs. This procedure replaces the three pairs of primers used by Cambien et al. (3) to amplify and determine the same polymorphisms (codons 10 and 25 are also amplified in the same PCR reaction) and the design and temperature optimization of eight ASOs. It also reduces the processing time per sample because the ASO method not only needs PCR amplification but also needs 6 h of hybridization, without taking into account the time for autoradiography. Digestion with restriction enzymes and polyacrylamide electrophoresis can be performed in 5 h. The present restriction enzyme method also avoids the use of radioactivity ($^{32}$P-labeled oligonucleotides).

Recently, a codon 10 polymorphism has been characterized by allele-specific amplification (5). Allele-specific amplification is based on two primers, each differing from the other in the terminal 3' nucleotide, which are specific for each allelic variant. Taq polymerase will not extend if a mismatch is present at the 3' end of the primer-template duplex. This method involves a limited effort because only one PCR reaction is necessary for each allele-specific primer. Direct visualization on agarose gels is the only postamplification processing. However, primer design and PCR conditions are critical because under some conditions, a single 3'-mismatched base permits amplification (6). Purine-purine and pyrimidine-pyrimidine mismatches are more refractory to extension than purine-pyrimidine mismatches; in such cases, additional mismatches can be introduced to destabilize the primer-template duplex and to increase specificity. Magnesium, dNTPs, Taq polymerase or primer concentrations, and the precise temperature of annealing must be carefully controlled to maintain the specificity of the reaction (7).

Another three polymorphisms were described by Cambien et al. (3) in the TGFB1 gene. A C→A transversion was detected at position −988; this could be considered as a variant because it was present in only 2 of 1000 individuals. A “C” insertion was also reported at position +72 in the 5'-untranslated region. This polymorphism is in mutual association with a codon 25 polymorphism (as shown by the strong linkage disequilibrium coefficient of −1.0 reported); therefore, we decided to exclude it and to analyze codon 25 polymorphisms. The +72 polymorphism is more difficult to detect than the C→G transition at codon 25, the latter being easily detected by the loss of an FseI site. This allows us to use only one pair of primers to amplify codons 25 and 10 in one PCR reaction. Another variant was reported in codon 263. In this case, a C→T transition produces a Thr$^{263}$→Ile substitution, the Thr allele being much more frequent, with only 6.7% of the subjects analyzed being heterozygous for this polymorphism. To our knowledge, there is no available enzyme capable of recognizing this substitution; therefore, the use of allele-specific primers or the design of a modified primer introducing an artificial restriction site (8) could be considered.

The genotyping system described here is simple, reliable, and relatively inexpensive when a large sample set needs to be genotyped, and it could be performed in laboratories without facilities for radioactivity. In the future, clinical studies on the TGFB1 polymorphisms could confirm suspicions of the crucial role of TGF-β1 and genetic predisposition to several fibrotic diseases.

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References


Analysis of Cell-free Epstein-Barr Virus-associated RNA in the Plasma of Patients with Nasopharyngeal Carcinoma, Kwok-Wai Lo,1 Y.M. Dennis Lo,2 Sing-Fai Leung,3 Yuen-Shan Tsang,1 Lisa Y.S. Chan,2 Philip J. Johnson,3 N. Magnus Hjelm,2 Joseph C.K. Lee,1 and Dolly P. Huang.2 (Departments of 1 Anatomical and Cellular Pathology, 2 Chemical Pathology, and 3 Clinical Oncology, Faculty of Medicine, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, New Territories, Hong Kong Special Administrative Region; * author for correspondence: fax 852 2637 6274, e-mail waisinhuang@cuhk.edu.hk)

Chen et al. (1) and Nawroz et al. (2) have reported that tumor-derived DNA is detectable in the plasma and serum of cancer patients and have opened up a new molecular approach for the early detection of malignancy. It is not known, however, whether circulating tumor-derived RNA is also present in plasma, because of the