Genetic Diagnosis by Polymerase Chain Reaction and Electrospray Ionization Mass Spectrometry: Detection of Five Base Deletion From Blood DNA of a Familial Adenomatous Polyposis Patient

Yasuhiro Naito
Department of Biochemistry, Hamamatsu University School of Medicine, Handacho, Hamamatsu, Shizuoka, Japan

Keiichiro Ishikawa and Yoshinori Koga
Laser-induced Reaction Laboratory, Department of Advanced Chemical Technology, National Institute of Materials and Chemical Research, Higashi, Tsukuba, Ibaraki, Japan

Toshihiro Tsuneyoshi
Department of Materials Science, Shizuoka Institute of Science and Technology, Toyosawa, Fukuroi, Shizuoka, Japan

Hideya Terunuma
Suruga Nishi Hospital, Nakane Shinden, Yaizu, Shizuoka, Japan

Ryuichi Arakawa
Department of Applied Chemistry, Faculty of Engineering, Osaka University, Machikaneyama-cho, Toyonaka, Osaka, Japan

A 5-base deleted mutation of adenomatous polyposis coli (APC) gene was detected by using electrospray ionization mass spectrometry of polymerase chain reaction (PCR) products. Genomic DNA was extracted from a familial adenomatous polyposis patient blood, and a 57-base pairs segment of APC gene was amplified by PCR. The PCR products were purified, digested with restriction endonuclease, purified, and determined by electrospray mass spectrometry. (J Am Soc Mass Spectrom 1997, 8, 737–742) © 1997 American Society for Mass Spectrometry

Genetic diagnosis has become an important area in medicine because it can determine whether one has specific gene mutations responsible for various hereditary or sporadic diseases. A variety of methods have been developed during the last two decades to detect a mutation by using the polymerase chain reaction (PCR) [1] (e.g., the PCR restriction fragment length polymorphism method (PCR-RFLP) [2, 3], the PCR-allele specific oligonucleotide method (PCR-ASO) [4], multiplex PCR [5], allele specific-PCR (AS-PCR) [6], the PCR-single stranded conformational polymorphism method (PCR-SSCP) [7], heteroduplex analysis [8], DNA sequencing [9, 10]). Most of these methods depend on gel electrophoresis to separate by size the target DNA from impurities or byproducts.

By using a polyacrylamide gel electrophoresis for DNA sequencing, which has the highest resolution of all gel electrophoresis-based methods for DNA analysis, however, we can determine the base length of DNA, but cannot distinguish one base substitution. This is the limit of resolution of the electrophoresis-based method [11–13]. Furthermore, electrophoresis-based methods are laborious, time-consuming, and sometimes require the use of radioactivity.

Mass spectrometry formerly could not be fully applied to the analysis of nucleic acids [14], because the negatively charged phosphodiester backbone is fragile under the usual ionization techniques and, therefore,

Address reprint requests and correspondence to Toshihiro Tsuneyoshi, Department of Materials Science, Shizuoka Institute of Science and Technology, 2200 Toyosawa, Fukuroi-City, Shizuoka 437, Japan.

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nucleic acids easily decompose into many fragments. Furthermore, the backbone has an affinity for nonvolatile cations, especially Na^+ and K^+ and, therefore, the mass peak of the nucleic acid is split into many satellite peaks. With recent development of more gentle ionization techniques such as electrospray ionization (ESI) [15–20] and matrix-assisted laser desorption ionization (MALDI) [21–27], it has become possible to determine higher molecular weight oligonucleotides (>10,000 Da) by mass spectrometry. It was also found in recent works that removal of nonvolatile cations is very important to obtain better mass spectra; replacement of sodium ion with ammonium ion followed by ethanol precipitation, use of a cation-exchange column for removal of the sodium ion, and the addition of triethylamine, imidazole, or piperidine for adsorbing sodium ion have made it possible to measure 50- to 100-mer synthetic oligonucleotides by ESI-mass spectrometry (MS) [28] or ESI coupled with Fourier-transform mass spectrometry (ESI-FTMS) [29–31].

Usually the minimum amount of DNA necessary for ESI measurements is 0.2 to 1 nmol. Because such a large amount of DNA is difficult to obtain directly from a human specimen, a large scale of PCR amplification is essential. Another major problem is that the PCR products have nonspecific molecular weights, because Taq polymerase transfers a nontemplated and unexpected nucleotide adduct to the 3′-end of each strand of the PCR products owing to its terminal deoxynucleotidyl transferase activity [32]. To avoid this problem, we designed PCR primers that included a restriction enzyme EcoRI recognition site at the 5′-end [33]. This technique is quite common in the recombinant DNA method in which the target DNA produced by PCR with primers including restriction enzyme recognition site is digested, for ligating it into circular vector DNA, which is also digested with the same enzyme. The PCR products obtained by this strategy were purified and digested by EcoRI to remove the nontemplated ends. An alternate method to avoid the nontemplated nucleotide adduct was demonstrated by using the Pfu DNA polymerase, which has more faithful polymerase activity than Taq [31].

Here, we demonstrate that a 5-base deletion of the APC gene can be accurately determined by ESI-MS from PCR amplified human blood DNA. The APC gene is one of the cancer suppressor genes, and germline mutation in it generally causes FAP, which is an autosomal dominant inherited disease, and confers a high risk of colon cancer [34–37] before the age of 40. This result shows that ESI-MS will be a powerful tool for genetic diagnosis.

Experimental

Sample Preparation

The sample preparation was described elsewhere [33]. Briefly, Genomic DNA was prepared from 1 mL of human whole blood [38]. The first PCR was performed with 100 ng of genomic DNA and 1 pmol of primer IC1 and IC2 in 100 μL solution that also contained 2.5 units of Taq polymerase, buffer, and deoxynucleotide triphosphate (dNTP) [39]. A second PCR was performed in 50 microtubes, each with one-fiftieth (0.2 μL) of the first PCR products solution, 10 pmol each of primer HT17 and HT18, which included the EcoRI restriction enzyme recognition sites at the 5′-end [33], dNTP, Taq DNA polymerase, and buffer together in a 100-μL solution for producing 57 bp (normal products) or 52 bp (5-bp deleted products) ds-DNA segments internally to the first PCR products. The PCR products were concentrated and purified by the phenol/chloroform extraction and the ethanol precipitation method [40]. The purified products were then digested with 2500 units of restriction enzyme EcoRI for 2 h at 37 °C. The digested products were purified by the phenol/chloroform extraction and the ethanol precipitation method and redissolved into 250 μL of pure water. The solution was mixed with an equivalent volume (250 μL) of 10 M ammonium acetate for 2 h at room temperature. Products were then purified by the ethanol precipitation method and redissolved into 250 μL of pure water again. This procedure was repeated three times.

ESI Measurement

ESI-MS spectra were obtained by an HX110/110A double-focusing tandem mass spectrometer with a 2048 channel array detector (JEOL, Tokyo, Japan). Samples were dissolved in aqueous 80% (v/v) acetonitrile containing 5% triethylamine at a final concentration of 1–5 pmol/μL. 50-μL aliquots of DNA preparations were introduced into the ion source at a flow rate of 2 μL/min. The spray needle was biased at −2.5 kV. The voltage between the extraction cone and the first skimmer element was usually set to 0 V. The source temperature was set to 70 °C.

Results and Discussion

To avoid the effect of terminal deoxynucleotidyl transferase activity, the EcoRI recognition sites were included at the 5′-end of oligonucleotide primers (HT17, HT18), and the PCR products were digested by EcoRI according to a strategy described previously [33]. Therefore, both ends of the PCR products, including the nontemplated nucleotide adducts, were removed, and the digested PCR products had a uniform base sequence and molecular weight. In a previous paper [33], we obtained mass spectrum of 49-bp PCR products from the blood DNA of a normal volunteer who has no 5-bp deletion mutation on the APC gene, showing an accurate measurement of the molecular masses (shown in Table 1). However, the thorough removal of the Taq

Table 1. Expected and measured masses of the PCR products with corresponding sequences

Expected PCR products	Expected mass	Measured mass
(Normal sense strand)		
5′-AATTCCCTGCAAATAGCAGAAATAAAAGAAAAGATTGGAACTAGGTCAG-3′	15,275.8	15,277.2 ± 1.8[a]
		15,275.2 ± 1.6[b]
3′-GGGACGTTTATCGTCTTTATTTTCTTTTCTAACCTTGATCCAGTCTTAA-5′	15,029.8	15,028.5 ± 4.4[a]
(Normal antisense strand)		15,029.0 ± 1.3[b]
(Deleted sense strand)		
5′-AATTCCCTGCAAATAGCAGAAATAAAAGATTGGAACTAGGTCAG-3′	13,693.8	13,693.2 ± 2.1
3′-GGGACGTTTATCGTCTTTATTTTCTAACCTTGATCCAGTCTTAA-5′	13,523.8	13,522.5 ± 2.3
(Deleted antisense strand)		

Upper and lower sequences show the normal and deleted double stranded PCR products, respectively. [a]Values were obtained from the normal DNA allele of the FAP patient. [b]Values were reported previously for the normal volunteer [33]. Repetitive sequence, which was affected from the deletion, was indicated by underline.

polymerase prior to the EcoRI treatment was essential to obtain a reasonable mass spectrum. A mass spectrum obtained from the EcoRI digested PCR products without thorough removal of the Taq polymerase is shown in Figure 1. In this case, the PCR products were concentrated and purified by ethanol precipitation without phenol/chloroform extraction. The rest of the sample preparation procedure is the same as that described in Sample Preparation. The measured masses of 16,508.4 ± 3.5 and 16,263.1 ± 3.0 Da from major peaks were far from expected values, 15,275.8 and 15,029.8. Furthermore, there was another group of peaks that give a measured mass value of 16,841.7 ± 2.0 Da. In the sample preparation procedure, PCR products as

```
5′-GGAATTCCCTGCAAATAGCAGAAATAAAAGAAAAGAT
   ||||||||||||||||||||||||||||||||||||||
3′-CCTTAAGGGACGTTTATCGTCTTTATTTTCTTTTCTA
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                    TGGAACTAGGTCAGAATTCC-3′
                    ||||||||||||||||||||
                    ACCTTGATCCAGTCTTAAGG-5′
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from primers HT17 and HT18 were digested with EcoRI as

```
5′-AATTCCCTGCAAATAGCAGAAATAAAAGAAAAGATTG
      ||||||||||||||||||||||||||||||||||
   3′-GGGACGTTTATCGTCTTTATTTTCTTTTCTAAC
```

```
                    GAACTAGGTCAG-3′
                    ||||||||||||
                    CTTGATCCAGTCTTAA-5′
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The digested products were then thought to be filled with dNTPs at the recessive end as

```
5′-AATTCCCTGCAAATAGCAGAAATAAAAGAAAAGATTG
   ||||||||||||||||||||||||||||||||||||||
3′-TTAAGGGACGTTTATCGTCTTTATTTTCTTTTCTAAC
```

```
                    GAACTAGGTCAGAATT-3′ (A)
                    ||||||||||||||||
                    CTTGATCCAGTCTTAA-5′ (B)
```

by the Taq polymerase, which still existed in the EcoRI digest. Furthermore, the filled (blunt end) products of nontemplated nucleotide G was added to the 3′-end of both strands as

```
 5′-AATTCCCTGCAAATAGCAGAAATAAAAGAAAAGATTG
    ||||||||||||||||||||||||||||||||||||||
3′-GTTAAGGGACGTTTATCGTCTTTATTTTCTTTTCTAAC
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```
                    GAACTAGGTCAGAATTG-3′
                    |||||||||||||||||      (C)
                    CTTGATCCAGTCTTAA-5′
```

The former two mass values were assigned to the sense [16,510.6; (A)] and antisense [16,264.6; (B)] strand of the "blunt end" products. The remaining value was assigned to a guanine adduct [16,839.8; (C)] to the sense strand of the "blunt end" products. The results indicate that Taq polymerase is slightly active even after the ethanol precipitation and has the terminal deoxynucleotidyl transferase activity [32]. If gel electrophoresis had been employed in the analysis, this unexpected phenomenon may have been missed. ESI-MS clearly detected this phenomenon, indicating one of the advantages of MS.

Next, genomic DNA, which was extracted from the blood of a FAP family member, was examined. The proband of the family carried a 5-bp deletion (AAAGA) at codon 1,309 of APC gene, which is often observed in the FAP genotypes, and it was already determined that

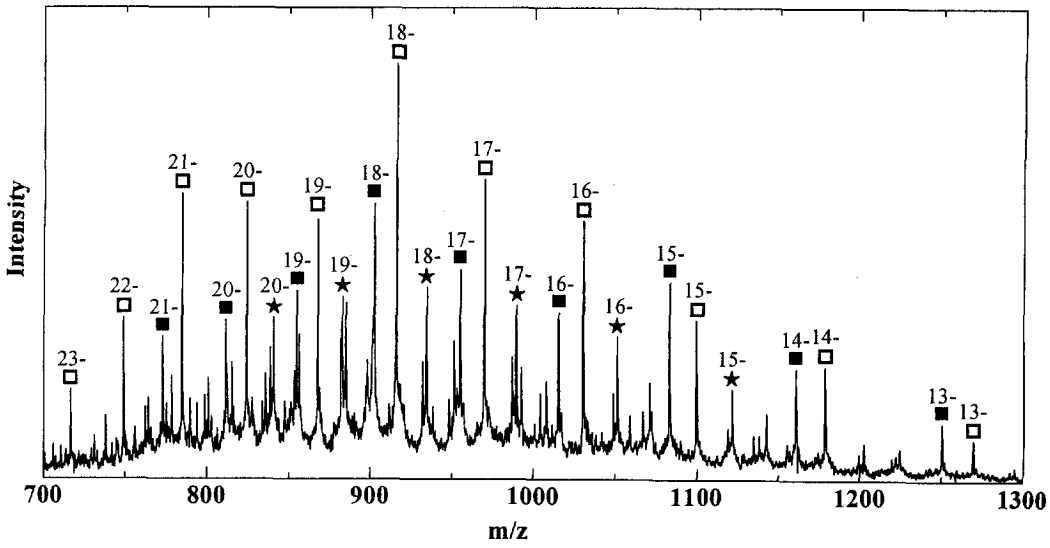

Figure 1. Mass spectrum of EcoRI digested PCR products without intensive removal of Taq polymerase. The sense and antisense strand of "blunt end" products that were derived from a filling up of the recessed ends of EcoRI digested PCR products with dNTPs were indicated with open and filled squares, respectively. An additional group of ion peaks, which was derived from a guanine adduct to the sense strand of the "blunt end" products, was indicated with a filled star.

the family member carried the deletion (mutation analysis of this family was described in detail elsewhere [39]). The proband inherited the mutation from an affected parent, so one allele of the subject is deleted and the other is intact. The heterozygosis is consistent with the mass spectrum (see Figure 2) that consists of four major components of ion peaks. The measured masses of 13,693.2 ± 2.1, 13,522.5 ± 2.3, 15,277.2 ± 1.8 and 15,028.5 ± 4.4 Da were assigned to a sense strand of the deleted allele, an antisense strand of the deleted allele, a sense strand of the normal allele and an antisense strand of the normal allele, respectively (Table 1). The expected masses of each fragment are 13,693.8, 13,523.8, 15,275.8, and 15,029.8 Da, respectively, corresponding closely to the measured mass. The spectrum obtained from the healthy individual in the previous

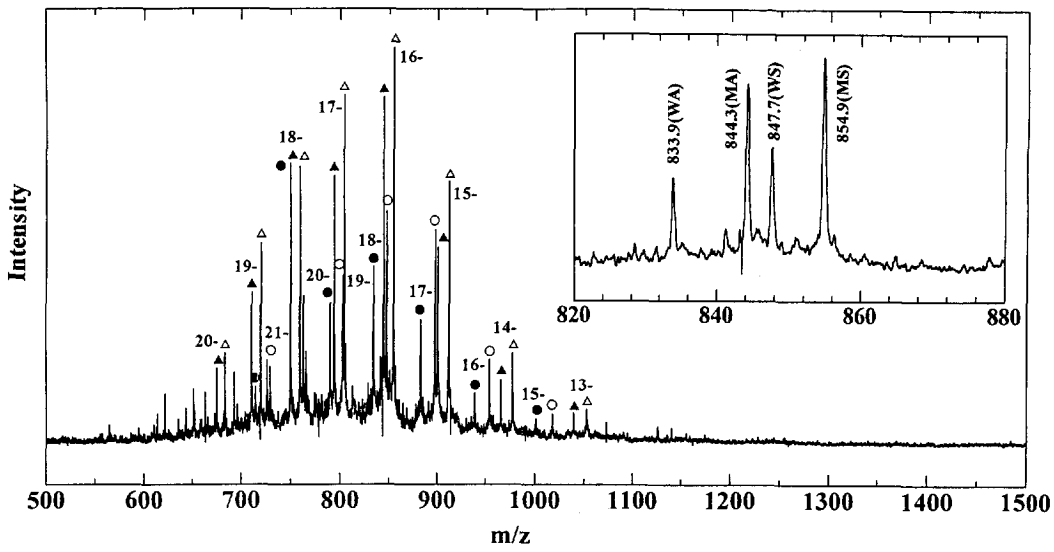

Figure 2. Mass spectrum of EcoRI digested PCR products amplified from an FAP family member. The sense and antisense strand of the digested PCR products from normal allele was indicated by open and filled circles, respectively. The sense and antisense strand from the deleted allele was indicated by open and filled triangles, respectively. The inset shows the fine structure of ion peaks that were assigned to the ion fragments with 18− charge from the normal (wild type) sense strand (WS) and normal antisense strand (WA), and the ion fragments with 16− charge from the deleted (mutant type) sense strand (MS) and deleted antisense strand (MA), respectively. See Table 1 for mass value comparison.

paper [33] serves as the control for the present study, and the control showed two accurate mass values corresponding to the sense and antisense strand of the normal allele. Thus, the PCR products from the healthy individual showed only two groups of mass peaks, while those from affected individual showed an additional two groups of mass peaks corresponding accurately to the deleted mass values. The measured mass differences between the normal allele and the deleted allele of 1583.9 ± 2.8 (sense strand) and 1506.0 ± 5.0 Da (antisense strand) also correspond closely to the expected masses of deleted segment of 1582.0 (AAAGA) and 1506.0 (TTTCT) Da, respectively. This does not mean that we could determine the sequence of the deleted part, but that we could only confirm the deletion.

In this communication, we have clearly demonstrated that ESI-MS allows precise measurement of PCR products and accurate detection of a 5-bp deletion, although it does not allow us to determine the sequence. The mass resolving power of ESI-MS seems to be sufficient to determine the difference in molecular weight of as low as 9 Da, which is the smallest difference in case of one base substitution (A to T), while the gel electrophoresis can only detect one nucleotide length difference at most. Recently, Doktycz et al. and Chang et al. reported genetic diagnosis by time-of-flight mass spectrometry and MALDI [41, 42], but the resolving power of their spectra was no better than that of gel electrophoresis.

Conclusion

We demonstrated the first application of PCR-ESI-MS to an actual genetic diagnosis. We detected a 5-base deletion mutation from the blood DNA of an FAP patient. However, it is difficult now to replace gel electrophoresis with mass spectrometry in the fields of genetic diagnosis and other DNA analysis, because the sample preparation procedure is laborious and the instrument is far more expensive than the gel-electrophoretic apparatus. However, mass spectrometry has many advantages including accuracy, rapidity, and ease of automation. In the near future, mass spectrometry will be a powerful tool for DNA analysis comparable to or superior to gel electrophoresis if the sample preparation is simplified and a specialized and inexpensive mass spectrometer for DNA analysis is developed.

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