Analysis of single nucleotide polymorphism sites in exon 4 of the p53 gene using high-performance liquid chromatography/electrospray ionization/ tandem mass spectrometry

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Three groups of four oligonucleotides with special single nucleotide polymorphism (SNP) sites in exon 4 of the p53 gene were analyzed with ion-pair, reversed-phase, high-performance liquid chromatography/electrospray ionization/tandem mass spectrometry. The retention order of four oligonucleotides with SNPs was C < G < A < T, regardless of whether the polymorphisms were at the 3′ end, the 5′ end, or the middle of the oligonucleotides. The charge state of the molecular ion affects the tandem mass spectra of the oligonucleotides. SNPs at the 3′ end can easily be identified from the fragmentation pattern of the 2- charge state, but not from the 3- charge state, especially from the w1 fragment. The single base may be taken as the symbol of the 5′ end SNP site derived from $[M-3H]^{3-}$, but not from the $[M-3H]^{2-}$ charge state. The oligonucleotides with SNPs in the middle were also determined from the $[M-2H]^{2-}$ precursor ion.

Keywords: single nucleotide polymorphisms (SNPs), high-performance liquid chromatography, electrospray ionization mass spectrometry, SNP site, retention order, product ion

Introduction

Single nucleotide polymorphisms (SNPs) are single-base changes that occur at specific positions in a genome.[1] They are powerful genetic markers for investigating the variability in the human genome and are particularly useful for mapping the genome, understanding and diagnosing diseases (including cancer) and revealing predispositions to side effects of drugs. Thus, discovering the link between SNPs and mutations is of great interest in contemporary life science.[2] SNPs are rapidly being mapped and archived (http://snp.cshl.org).[3] More than two million are already in the public domain. With an increased emphasis on genotyping SNPs in disease association studies, the genotyping platform of choice is constantly evolving. The development of more specific SNP assays and appropriate genotype validation applications is becoming critical for indicating ambiguous genotypes.[4] SNPs in cancer-related genes can act as low-risk genetic factors for the development of cancer. SNPs have also been shown to influence the efficacy and toxicity of various cytotoxic agents used in treating cancer.[5] SNPs and mutations commonly exist in the p53 gene that encodes the tumor-suppressor protein. It is known that mutations also appear most frequently as changes in single nucleotides. SNPs occur at specific nucleotide positions, whereas mutations may be found in various locations within genes or large regions of genes. In detecting an SNP, it is only necessary to analyze small pieces of deoxyribonucleic acid (DNA) that contain the known nucleotide substitution. One of the most frequent SNPs within the p53 suppressor gene is found in exon 4, which was taken as our target sequence.[6]

A number of methods have been developed for analyzing SNPs, including polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP), real-time PCR and denaturing high-pressure liquid chromatography (DHPLC). Although SNPs can also be discovered by aligning multiple sequences of publicly available data, recent studies indicate that only a small fraction of them can be found in this way. Uncommon SNPs are often missed.[4,7–9]

Mass spectrometry (MS) has recently been proven useful for analyzing nucleic acids for SNP genotyping purposes by distinguishing the molecular weight of four bases, even though they differ by only 9 Da.[7,10–15] Electrospray ionization (ESI) has potential for use in combination with tandem MS (MS/MS) to obtain structural information when two precursor ions have the same mass but different sequences, and can be used for identifying resequencing or unknown polymorphisms. The direct combination of liquid chromatography on-line mass spectrometry and ESI has been an important technology for characterizing proteins and nucleic acids.[16–19] Because oligonucleotides can be desalted and separated by high-resolution high-performance liquid chromatography (HPLC) before mass spectrometry, HPLC-ESI-MS/MS offers the greatest selectivity and specificity for mixtures of oligonucleotides. HPLC-ESI-MS/MS has the advantage of also providing structural specificity.[20] Even so, this technique has rarely been used to measure the characteristics of SNP sites in the p53 gene.

Since previous research has reported that a key factor in determining the fragmentation of product-ion spectra is the charge state of precursor ions, it is important to choose the appropriate charge state for the primary fragmentation at the optimal collision energy. The effect of charge state in determining the SNP site using HPLC-ESI-MS/MS has rarely been reported to date.

We have recently used reversed-phase, ion-phase, high performance liquid chromatography/electrospray ionization mass spectrometry (RP-IP-HPLC/ESI-MS) to study the characteristics of G-rich, T-rich, and AT-rich oligonucleotides and to analyze the methylation of DNA.[21–24] The research reported here demonstrates how HPLC-ESI-MS/MS can be used to discriminate between three groups with different SNP sites in exon 4 of the p53 gene. The method is simple, rapid, and accurate at various charge states of oligonucleotides.

Experimental

Chemicals and materials

Oligonucleotides were obtained from Asia Biochemistry (Shanghai, China) and were used as received. The sequences that were investigated are listed in Table 1.

Triethylamine (TEA, 99.5%), glacial acetic acid (HAc 99.99%) and HPLC-grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Water was purified with an Elix-Milli-Q system (Millipore Corp., Bedford, MA, USA). 1,1,1,3,3,3-hexafluoro-2-propanol was obtained from Dupont (Delaware, USA).

The 100-mM triethylammonium acetate buffer, pH 7, was prepared by mixing 7.2 mL of TEA and 2.98 mL of acetic acid in 400 mL of water and then carefully adjusting the pH to seven with TEA or acetic acid. After adjusting the volume to 500 mL, the concentration of triethylammonium acetate was 100 mM.

The mobile phase was filtered through a 0.22 μm film before being used in the HPLC analysis.

Instrumental analysis

All samples were analyzed with an Agilent 1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled to an API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA). The liquid chromatograph was equipped with an on-line vacuum degassing system, a quaternary pumping system, an autosampler and a variable wavelength detector. The chromatographic separation used a C18 column (150 × 3.0 mm i.d., 3.0 μm particle size, Zorbax 300SB, Agilent Technologies, Palo Alto, CA, USA) at room temperature. Three mobile phases at a flow-rate of 300 μL min^{-1} were used in the analysis, under conditions given in the figure captions. The concentration of the single oligonucleotide used in the experiments was 0.2 nmol μL^{-1}. The injection volume was 1 μL and UV detection used a wavelength of 260 nm. The LC effluent was introduced into a Turbon IonSpray interface without splitting. Electrospray mass data were acquired in the negative-ion mode with a spray voltage of −4 kV and a declustering potential of −100 V. The source temperature was 400°C. Nitrogen was used as the curtain gas (setting 16), nebulizer gas (setting 20) and turbo gas (setting 20). MS/MS was performed using nitrogen as collision gas (CAD gas setting

Table 1. Characteristics of SNP sites investigated here.

Site	Sequence	Molecular weight (Da)	
		Theoretical	Experimental
3-C	5′-CTGTC*C*-3′	1743.2	1743.9
3-G	5′-CTGTC*G*-3′	1783.2	1783.1
3-A	5′-CTGTC*A*-3′	1767.2	1767.6
3-T	5′-CTGTC*T*-3′	1758.2	1758.3
5-C	5′-*C*TGTCC-3′	1743.2	1742.8
5-G	5′-*G*TGTCC-3′	1783.2	1783.4
5-A	5′-*A*TGTCC-3′	1767.2	1766.9
5-T	5′-*T*TGTCC-3′	1758.2	1758.9
m-C	5′-CT*C*TCC-3′	1703.2	1702.7
m-G	5′-CT*G*TCC-3′	1743.2	1743.8
m-A	5′-CT*A*TCC-3′	1727.2	1727.5
m-T	5′-CT*T*TCC-3′	1718.2	1718.9

8). The mass spectrometer was operated at unit–mass resolution for both Q1 and Q3. The data were acquired with Sciex Analyst software, version 1.3.1 (Applied Biosystems, Foster City, CA, USA).

Results and discussion

Retention behavior of SNPs at three sites in exon 4 of the p53 gene using IP-RP-HPLC

IP-RP-HPLC has been used extensively for separating mixtures of oligonucleotides.[25,26] Their retentions are influenced by size, sequence, base composition, and hydrophobicity. In our previous study, retention times of G-rich and T-rich oligonucleotides were compared by relying on the hydrophobicity of oligonucleotides by HPLC. Previous work has focused on the two SNP sites at the 3′ end, but not on the site at the 5′ end.[27] This is the first time that four SNPs at the 5′ end have been determined by HPLC-ESI-MS/MS.

The retention times were in the order C<G<A<T when the SNP sites were at the 5′ end. Table 2 shows the HPLC spectrum of four oligonucleotides with different bases at the 5′ end as eluted by the TEAA buffer system at room temperature. Since the only difference in the four oligonucleotides was the single base, their hydrophobic character controlled their retention time. Table 2 showed that the difference in retention time was much greater between 5-C and 5-T than between 5-C and 5-G and that the largest difference was between 5-C and 5-T. In other words, the more hydrophobic the base, the greater was the retention time. This result agreed with other studies.[27,28]

Four oligonucleotides with SNP sites at the 3′ end were investigated with ion-pair, reverse-phase, high-performance liquid chromatography. Table 2 indicates that retention times were in the order C<G<A<T (at the 3′ end). The order was the same as with the SNPs at the 5′ end. As in the 5′ case, the difference in retention time was much higher between 3-C and 3-T than between 3-C and 3-G and the largest difference was between 3-C and 3-T. Again, this was due to the differences in hydrophobicities of the bases at the 3′ end. Adding C or G did not increase the retention time as much as adding A or T. This result was similar to that obtained by Gilar *et al.*, who used the hetero-oligonucleotide ladder with HPLC.[24] The position of SNPs does not affect retention time as much as hydrophobicity does.

Few studies have focused on the retention time of SNPs in the middle of oligonucleotides. To compare the retention order of these SNPs with those at the 3′ and 5′ ends, four oligonucleotides with SNPs in the middle were analyzed with HPLC, as described above. The retention times again fell in the order C<G<A<T, the same as for the two previous groups of oligonucleotides (Table 2). This result differed from other reports. For example, Gilar *et al.*[24] reported that different bases at the middle position did not affect the retention behavior. The discrepancy may be because the sequence of oligonucleotides differed. In the present experiment, single-base C-to-T transformation (m–T) has more hydrophobic T. Composition T is higher than others, so that the retention time is longer than the others' transformation.

Characterization of SNPs at three sites in exon 4 of the p53 gene by ESI-MS/MS

ESI-MS has played an important role in analyzing oligonucleotides because of its rapidity and accuracy. MS/MS has been used successfully to characterize oligonucleotides and identify their variations in sequence. It is known that the parent ions in ESI are often multiply charged, which would affect the fragmentation patterns.[29–33]

In order to investigate the MS/MS characteristics of oligonucleotides with SNPs, negative ESI-MS/MS was used with four oligonucleotides of 6-mers. There is a distribution of multiply charged molecular ions with few adduct ions present. The charge states, typically, ranged from 2- to 4-, and were 2- and 3- in the present experiments. The abundance of $[M–4H]^{4-}$ was too low to allow the product ions to be identified with our instrument.

The MS/MS spectra of 5-C in the charge states $[M–2H]^{2-}$ and $[M–3H]^{3-}$, obtained with collision energies of −30 and −20 eV, respectively (Figure 1), show that SNP sites at the 5′ end were easier to identify with $[M–3H]^{3-}$ than with $[M–2H]^{2-}$. The optimal collision energy was set to maintain the relative abundance of the precursor ions at a set level. Higher charges will more easily form diagnostic fragments that can be identified from the different base of oligonucleotides, while lower states will form more sequence fragments. According to the fragmentation nomenclature of McLuckey,[33] the common fragment ions are the 3′ end w series and the 5′ end a-B series. C- is the predominant ion in the spectrum from $[M–3H]^{3-}$, a2-B from $[M–2H]^{2-}$. The pair ion of a2-B and w4 indicates that guanine has cleaved at the lower charge state. The sequence fragments formed, derived from the 2- charge state, become the main reaction channel. The same result (that the SNPs were at the 5′ end) was found for the other three oligonucleotides (data not shown).

The other three oligonucleotides with SNPs at the 5′ end were studied with tandem mass spectrometry. Figure 2 shows that, in each of the MS/MS spectra for the $[M–3H]^{3-}$ charge state, the major fragment was the base with a single missing proton, although sequence fragments can be observed. G-, C-, A-, and T- can easily be obtained and identified from the
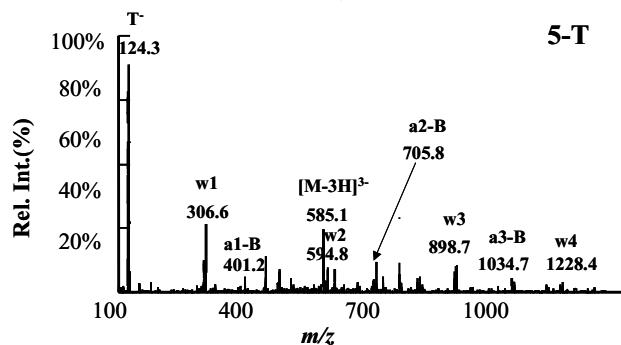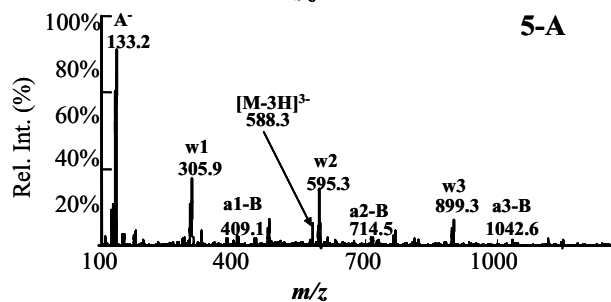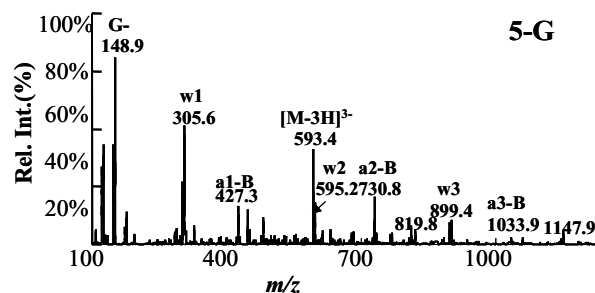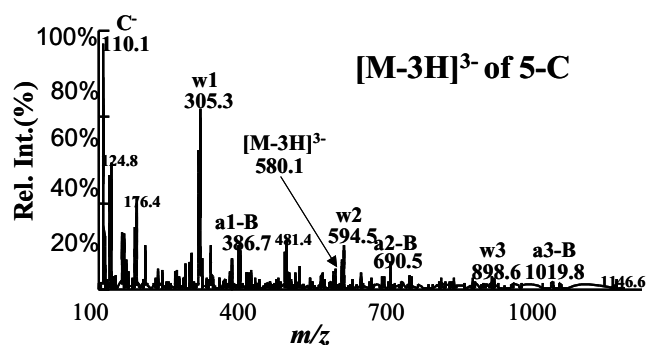
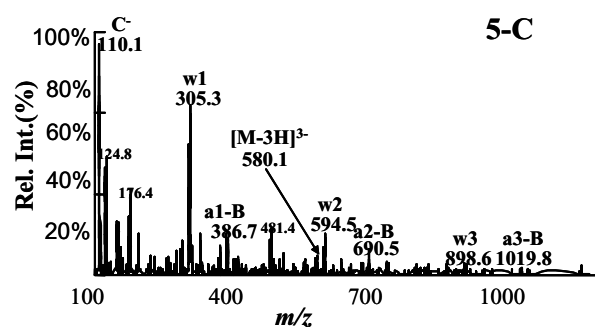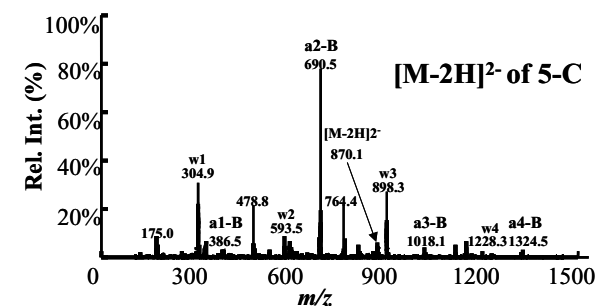| No·*	Retention time (min)		
	5′ end	3′ end	Middle
C	7.09	7.2	9.11
G	8.26	8.07	11.12
A	13.14	10.24	11.67
T	13.74	10.29	18.97

Figure 1. MS/MS spectra of the 5-C (5′-CTGTCC-3′) oligonucleotide from its 2- and 3- charge state at −30 and −20 eV collision energy, respectively.

Figure 2. MS/MS spectra of the 5-C, 5-G, 5-A and 5-T (5′-CTGTCC-3′; 5′-GTGTCC-3′; 5′-ATGTCC-3′; 5′-TTGTCC-3′) oligonucleotides from its 3- charge state at −20 eV collision energy.

MS/MS spectra. Fragments of the a-B series, which could determine the different bases at the 5′ end, are also observed. The intensity of a-B ions is weaker than for single bases. It is evident that the position of the SNPs could be determined from these major single-base ions. Furthermore, w-series ions are almost identical for the four oligonucleotides, showing that the 3′-end base is the same.

These results are the first determinations of the 5′-end SNPs at higher charge states. Luo *et al*. reported that although several mechanisms had been proposed for the fragmentation of oligonucleotides,[34,35] none of them fully explain all the reaction channels observed in different oligonucleotides.[29] In fact, the current study shows the interesting result that the trend of loss of nucleobases at a higher charge state from the 5′-end of oligonucleotides is almost the same. Therefore the fragmentation behavior as a function of charge state determined with tandem mass spectrometry is very useful for identifying SNPs at the 5′ end.

On-line ESI-MS was applied to four oligonucleotides with SNPs at the 3′ end. The charge states also ranged from 2- to 4-, but were mainly restricted to 2- and 3-. The spectra differed at [M–2H]$^{2-}$, but were similar at [M–3H]$^{3-}$. This result differed from that for the 5′-end polymorphisms. So the [M–2H]$^{2-}$ was used to provide the precursor ions for the MS/MS experiment. w1 was 304.9, 344.8, 329.0 and 320.0 in the MS/MS spectra (Figure 3). The SNP site was easily identified from the w1 ions. It agreed with our previous report, in which the oligonucleotides with 3′-end SNPs were 20 lengths.[21] The a-B series ions were almost identical in the present experiment.

The MS/MS spectra were similar to those with SNPs at the 3′ end. Therefore, [M–2H]$^{2-}$ precursor ions were chosen for the MS/MS experiment. Table 3 and Table 4 show the product ions relative intensity of four oligonucleotides of 2- and 3- charge states with middle polymorphisms. Table 3 indicates that among four oligonucleotides, complementary a-B-type and w-type ions can be used to identity the location of the nucleobase in the sequence due to w1 to w3 and a1-B to a2-B being the same, while the w4, a3-B and a4-B are different. The major ions were a2-B and w3 of m-C, which indicated that C in the middle forms sequence fragments
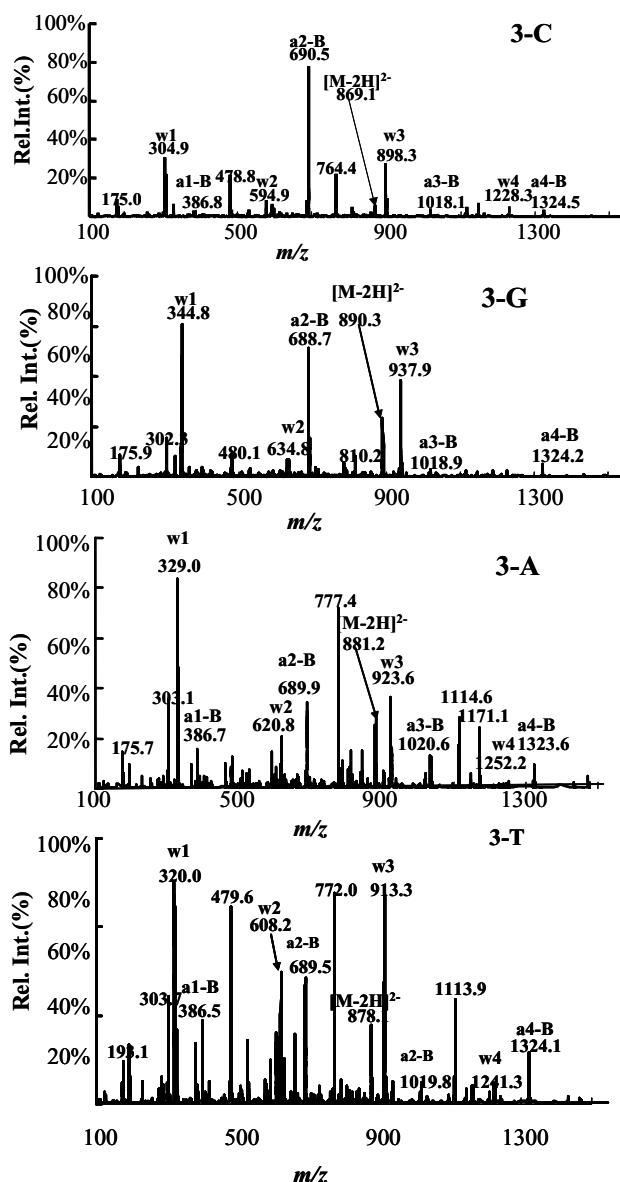
Figure 3. MS/MS spectra of the 3-C, 3-G, 3-A and 3-T oligo-nucleotides from its 2- charge state at −30 eV collision energy. (5′-CTGTCC-3′; 5′-CTGTCG-3′; 5′-CTGTCA-3′; 5′-CTGTCT-3′).

more easily that when at the end. In the MS/MS ion relative intensity of m-G oligonucleotides, the predominant ion was a2-B, which showed the loss of base G. Otherwise, w1 and w2 are the major ions formed by loss of C. The A and T bases are more difficult to lose to form sequence fragments than are C and G. As can be seen from the tables, SNP sites in the middle of oligonucleotides were readily identified with $[M-2H]^{2-}$ as the precursor ion.

Conclusion

Twelve oligonucleotides with different SNP sites in exon 4 of the p53 gene were determined with HPLC-ESI-MS/MS.

Table 3. Relative intensity of product ions of the m-C, m-G, m-A and m-T oligonucleotides from its 2- charge state at −30eV collision energy.

Fragments	Relative intensity of 2- charge state			
	m-C	m-G	m-A	m-T
α1-B	8.43	1.3	11.2	1.8
α2-B	73.8	100	28.4	10.6
α3-B	1.2	5.2	3.4	21.3
α4-B	10.1	4.2	12.2	16.8
W1	4.68	39.4	100	100
W2	56.5	8.5	44	62
W3	100	35.2	30.2	36
W4	1.2	1.9	2.6	3.7
A-	0	0	0.8	0
T-	2.1	1.8	2	2.5
C-	1.3	2.6	3.2	1.2
G-	0	2.3	0	0

The retention order of four oligonucleotides with SNPs was C<G<A<T within defferent sites. SNPs in the 3′ end are easily identified from the fragmentation pattern of the 2-charge state, but not from the 3-state, especially from the w1 fragment. The single base may be taken as the symbol of the 5′ end site derived from $[M–3H]^{3-}$, but not from the $[M–2H]^{2-}$ state.

Table 4. Relative intensity of product ions of the m-C, m-G, m-A and m-T oligonucleotides from its 3- charge state at −30 eV collision energy.

Fragments	Relative intensity of 3- charge state			
	m-C	m-G	m-A	m-T
α1-B	8.43	1.3	5.6	1.8
α2-B	19.7	21.6	7.3	8.6
α3-B	1.2	2.6	1.8	13.8
α4-B	3.9	2.1	9.7	10.2
W1	4.6	11.6	16	21
W2	12.7	3.2	16.2	20.1
W3	26	8.6	12.7	22.9
W4	1.2	1.9	0.6	1.8
A-	0	0	39.8	0
T-	30.1	18.6	12.7	49.2
C-	100	100	100	100
G-	0	26.3	0	0

Acknowledgments

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