Rapid and Simple Detection of Hot Spot Point Mutations of Epidermal Growth Factor Receptor, BRAF, and NRAS in Cancers Using the Loop-Hybrid Mobility Shift Assay

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A simple and rapid method to detect the epidermal growth factor receptor hot spot mutation L858R in lung adenocarcinoma was developed based on principles similar to the universal heteroduplex generator technology. A single-stranded oligonucleotide with an internal deletion was used to generate heteroduplexes (loop-hybrids) bearing a loop in the complementary strand derived from the polymerase chain reaction product of the normal or mutant allele. By placing deletion in the oligonucleotide adjacent to the mutational site, difference in electrophoretic mobility between loop-hybrids with normal and mutated DNA was distinguishable in a native polyacrylamide gel. The method was also modified to detect in-frame deletion mutations of epidermal growth factor receptor in lung adenocarcinomas. In addition, the method was adapted to detect hot spot mutations in the B-type Raf kinase (BRAF) at V600 and in a Ras-oncogene (NRAS) at Q61, the mutations commonly found in thyroid carcinomas. Our mutation detection system, designated the loop-hybrid mobility shift assay was sensitive enough to detect mutant DNA comprising 7.5% of the total DNA. As a simple and straightforward mutation detection technique, loop-hybrid mobility shift assay may be useful for the molecular diagnosis of certain types of clinical cancers. Other applications are also discussed. (*J Mol Diagn* 2006, 8:504–512; DOI: 10.2353/jmoldx.2006.060030)

Rapid and accurate detection of mutations in various cancer-related genes has become increasingly important to provide molecular diagnostic information about clinical cancers. For example, information of the mutated states of particular genes is crucial for successful chemotherapy with certain gene-targeting drugs. Namely, gleevec (imatinib) has been shown to be effective for gastrointestinal stromal tumor with specific mutations in KIT^{1,2} and PDGFRA,³ as well as for chronic myelogenous leukemia carrying the chimeric gene BCR/ABL1.⁴ Recently, a subset of lung adenocarcinomas with specific mutations in epidermal growth factor receptor (EGFR) has been reported to respond remarkably well to Iressa (gefitinib).^{5–9}

To detect mutations in these and other oncogenes, there are several long-standing methods available such as direct sequencing of polymerase chain reaction (PCR)-amplified DNA, single-strand conformation polymorphism (SSCP),¹⁰ SSCP/duplex analyses,¹¹ mutant allele-specific amplification,¹² and denaturing high performance liquid chromatography.^{13,14} These techniques have been successfully applied for the detection of mutational changes in various cancer genes. Each method has its own advantages as well as disadvantages or difficulties in practical situations. Direct sequencing of heterozygous point mutations and deletions may produce results requiring sophisticated data analysis for heterozygous mutations, especially in the presence of contaminating normal tissue DNA. SSCP, widely used for its simplicity, requires strict temperature control during a long electrophoretic time and radiolabeling in standard detection. SSCP/duplex and denaturing high performance liquid chromatography analyses necessitate sophisticated separation equipment such as capillary electrophoresis or high performance liquid chromatography with temperature control. Mutant allele-specific amplification requires several primers with mutational sites at the 3' ends to discriminate the mutational base changes by the lack of polymerase extension beyond the mis-

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matched end, and occasional read-through can cause ambiguous results.

Heteroduplex analysis using universal heteroduplex generator (UHG) technology^{15,16} is based on the retarded mobility in native polyacrylamide gel electrophoresis (PAGE) of a heteroduplex between the test PCR fragment and the PCR fragment termed the heteroduplex generator. Heteroduplex generators contain small deletions in the vicinities of mutational sites and generate four kinds of heteroduplexes with mutant and normal strands by hybridization, which are differentiated by the mobility changes. The method was applied to detect point mutations in sickle-cell diseases and phenylketonuria. Recently, UHG technology was adapted for detection of point mutations in NRAS at codons 12, 13, and 61.^{17,18} In UHG technology, band patterns of four different retarded bands in PAGE are analyzed to determine mutational states. We simplified UHG technology by using single-stranded oligonucleotides with internal deletions as the generators of the loop-bearing heteroduplexes. This modification yields two bands for heterozygosity and one band for homozygosity, enabling more straightforward data analysis. Our method, designated the loop-hybrid mobility shift assay (LH-MSA), was developed to detect the point mutation L858R of EGFR exon 21 in lung adenocarcinoma that is associated with responsiveness to gene-targeted kinase inhibitors such as gefitinib. Adaptations of LH-MSA to detect in-frame deletions of EGFR exon 19 in lung adenocarcinoma and hot spot point mutations of BRAF and NRAS in thyroid carcinoma are also described.

Materials and Methods

DNA Preparations and Plasmid Clones

DNA from fresh tumor tissues (16 cases of lung adenocarcinoma, 25 cases of papillary thyroid carcinoma, and 19 cases of follicular thyroid carcinoma) was prepared according to standard protocols after obtaining informed consent. Formalin-fixed, paraffin-embedded (FFPE) tissue sections of lung adenocarcinoma and papillary thyroid carcinoma were also used for preparing DNA as follows. Thin-sectioned tissues (15 to 20 μm thick) were deparaffinized with xylene followed by ethanol series and air-dried. Tumor tissues, identified in the hematoxylin and eosin-stained serial sections (2 μm), were applied with a pinpoint solution (Pinpoint slide DNA isolation system; Zymo Research, Orange, CA), air-dried, and cut out together with the overlaid dried film of the pinpoint solution. The excised tissues (3-mm square) were digested in proteinase K buffer solution at 55°C for 4 hours, heat inactivated at 95°C for 15 minutes, and used as PCR template directly, or after purification with a spin column.

PCR-amplified DNA fragments were ligated to the TOPO-TA ligation vector pCR4TOPO (Invitrogen, Carlsbad, CA), electroporated into *Escherichia coli*, and cloned. Cloned bacterial cells were suspended in lysis solution (CloneChecker; Invitrogen), heat-lysed at 98°C for 30 seconds, and used as the cloned plasmid DNA

solutions. DNA of the cloned plasmids was amplified with Phi29 polymerase (GE Health Care Bio-Science, Piscataway, NJ) and used for sequencing the inserts of tumor DNA fragments (CEQ8000 sequence analysis system; Beckman Coulter, Fullerton, CA). Direct sequencing of the PCR products from EGFR exon 21 was performed as described by Lynch and colleagues.⁶

Loop-Hybrid Formation in the LH-MSA

The LH-MSA consisted of two parts: hybridization of PCR products to the loop-hybrid generator (LH-G) probes made from synthetic oligonucleotides (70 to 99 mers) to generate loop-hybrids (Figure 1A) and analysis of mobility shifts of the loop-hybrids after native PAGE as described below. Nucleotide sequences of PCR primers and LH-G probes used to detect point mutations (7R, 18R, 9F, and 10K) and deletions (19JWTF) are described in Table 1. Each LH-G probe was designed to overlap with one of the PCR primer pairs at the 5' end, and a stretch of up to 18 nucleotides was deleted in the region adjacent to the mutational hot spot for detection of point mutations. To detect deletion mutations in EGFR exon 19, the LH-G probe (19JWTF) was an oligonucleotide with no internal deletion that extended 26 nucleotides beyond the region of deletion mutations so that a loop forms only when it hybridizes with PCR products containing a deletion in EGFR exon 19 (Figure 3A). LH-G probes were purified with high performance liquid chromatography. PCR was performed with Accuprime *Taq* polymerase containing primer-template hybridization-enhancing reagent (Invitrogen). Generation of loop-hybrids was conducted at the end of the PCR amplification cycles by adding a specific LH-G probe into the PCR reaction solution at a final concentration of 500 nmol/L, ie, in large excess to the initial concentration of primer pairs (200 nmol/L). The mixture was then subjected to the loop-hybrid formation (LH-F) steps, consisting of 1) denaturation at 94°C for 2 minutes, 2) hybridization of the LH-G probe to the complementary strand at 55°C for 15 seconds, and 3) extension of the 3' end of the LH-G probe in loop-hybrids by *Taq* polymerase at 68°C for 4 minutes. After the LH-F steps, reaction products were analyzed by PAGE to detect migration shift of the loop-hybrid bands by the mutations.

Detection of Loop-Hybrid DNA with PAGE

PCR products subjected to LH-F steps in the presence of LH-G probes were separated by electrophoresis in a native 10% polyacrylamide gel (6 cm $\times$ 6 cm preformed compact gels; ATTO Inc., Tokyo, Japan) in Tris-glycine buffer (37.5 mmol/L Tris, 288 mmol/L glycine) at 20 mA for 30 minutes at room temperature. Using a native 8% polyacrylamide gel in TBE buffer (89 mmol/L Tris, 89 mmol/L boric acid, 2 mmol/L ethylenediaminetetraacetic acid), electrophoresis of the loop-hybrid DNA performed at 10 mA for 1 hour at room temperature yielded equivalent results. After electrophoresis, gels were stained for 10 minutes with SYBER Green I (Cambrex Bio Science, Rockland, ME) diluted to 1/10,000 in distilled water and

Table 1. PCR Primers and LH-G Probes Used for Detection of Mutations in EGFR, BRAF, and NRAS

Gene	Mutation	Amplicon	PCR primer [†]		LH-G probe [‡] (probe name)				
EGFR	In-frame deletion in exon 19	212bp	(F)	GGACTCTGGATCCCAGAAGGTG	GGACTCTGGA	TCCCAGAAGG	TGAGAAAGTT		
			(R)	CATTTAGGATGTGGAGATGAGC	AAAATTCCCG	TCGCTATCAA	GGAATTAAGA		
EGFR	Point mutation in exon 21 at L858	161bp	(F)	GGCATGAAC TACTTGGAGGAC	GAAGCAACAT	CTCCGAAAGC	CAACAAGGAA		
					ATCCTCGAT	(19JWTF)			
					CTTACTTTGC	CTCCTTCTGC	ATGGTATTCT		
					TTCTCTTCCG	CACCCAGCAG	***** <u>A</u> GC		
					(R)	CTTACTTTGCCTCCTTCTGCATG	CCAAAATCTG	TGATCTTGAC	ATGCTGCG
							(7R)		
CTTACTTTGC	CTCCTTCTGC	ATGGTATTCT							
TTCTCTTCC*	*****	***** <u>A</u> GC							
CCAAAATCTG	TGATCTTGAC	ATGCTGCG							
BRAF	Point mutation in exon 15 at V600	157bp	(F)	ATTTCTTCATGAAGACCTCACAG	ATTTCTTCAT	GAAGACCTCA	CAGTAAAAAT		
					AGGTGATTTT	GGTCTAGCTA	CAGT*****		
NRAS	Point mutation at 061	149bp	(F)	GTGAAACCTGTTTGTGGAC	***ATGGAGT	GGGTCCCATC	AGTTTG		
					(9F)				
					GTGAAACCTG	TTTGTGGAC	ATACTGGATA		
					CAGCTGGACA	*****	AGTGCCATGA		
					(R)	CCTGTAGAGGTTAATATCCG	GAGACCAATA	CATGAGGACA	GG (10K)

[†](F), forward primers; (R), reverse primers.

[‡]*, deleted nucleotides; underlined nucleotides indicate the hot spots of mutations to be detected.

placed in deionized water to remove excess dye. After the staining, DNA was detected with a laser-scanning imager (STORM860; GE Health Care Bio-Science) using 450-nm excitation and a 520-nm long path filter. A 100-bp ladder (Promega, Madison, WI) was used to distinguish the bands of loop-hybrid DNA showing retarded migration from the homoduplex bands showing size-dependent migration.

Estimation of Mutant Copy Numbers with LH-MSA

Plasmid clones of PCR products from the L858R mutant and normal alleles of EGFR exon 21 were mixed together at mutant to normal ratios ranging from 1 to 0.05. After PCR of these mixed samples with Accuprime *Taq* High Fidelity (Invitrogen), an aliquot was examined directly with LH-MSA using the 18R LH-G probe to determine the sensitivity of mutation detection and the remaining portion was used for TOPO-TA ligation and cloning as described above. Forty-eight to ninety-six bacterial clones were genotyped with LH-MSA using the 18R LH-G probe to estimate copy numbers of the mutant allele in PCR products from the mixed samples. No mutant was found in the 48 clones of the normal control. Dideoxy sequencing of the mixed plasmid DNA was performed as described above.

Results

Detection of the EGFR Hot Spot Mutation L858R in Lung Adenocarcinoma Using LH-MSA

For simple and easy detection of the point mutation at L858 in EGFR exon 21, a method similar to the universal heteroduplex generator technology^{15–18} was developed. As illustrated in Figure 1A, the heteroduplex with a loop (hereafter referred to as loop-hybrid) can be generated by hybridization of the PCR product from EGFR exon 21

with a synthetic oligonucleotide (LH-G probe) having a stretch of nucleotides deleted adjacent to the mutation hotspot at L858. A series of LH-G probes expected to generate loops of various nucleotide lengths (Figure 1B) was examined. Migration of the loop-hybrids in PAGE was markedly retarded compared to the size-dependent migration of the homoduplex, and the degree of retarded mobility depended on the size of loop in the loop-hybrid (Figure 1, B and C). The loop-hybrid band of the mutant allele was markedly shifted from that of the normal allele when the same LH-G probe was used (Figure 1B). The shift was particularly pronounced for the LH-G probes 4R, 7R, 14R, and 18R. Faint secondary bands were visible beside the major LH band when the LH-G probes 3R, 6R, and 8R were used. DNA from the retarded bands excised from the gel was amplified with PCR and cloned into plasmids. When these clones were sequenced, both the original PCR fragment and the derivative of the LH-G probe used to generate the loop-hybrid were identified (data not shown), confirming our model (Figure 1A). When the faint secondary band produced by LH-G probe 8R was similarly analyzed, a mutation (one base deletion five bases upstream of the internally deleted site) was detected in the sequence corresponding to the 8R LH-G probe, whereas the expected sequence of 8R was found in the main loop-hybrid band. Therefore, the faint secondary band seemed to be generated by a contaminating mutant form of the 8R LH-G probe. Purification of LH-G probes with PAGE may be necessary to reduce such inadvertent contaminants. The LH-G probes 7R and 18R showed single, discrete loop-hybrid bands, well distinguishing the mutant from the normal allele and distinctly separated from the homoduplex band. Therefore, these LH-G probes were adopted in the following LH-MSA analysis to detect the L858R mutation in EGFR exon 21. Heterozygous mutations are detected as the double bands of the normal and the shifted mutant loop-hybrid bands.

Figure 1. Detection of the hot spot point mutation L858R (CTG>CGG) of EGFR exon 21 by LH-MSA using various LH-G probes. **A:** Schematic representation of loop-hybrids (LH) generated by an LH-G probe (G) in the PCR product heterozygous for a point mutation. Homoduplexes of the normal and mutant alleles are produced by polymerase extension of the hybridized PCR primer (p). The broken line indicates the 3' region of the LH-G probe to be extended by *Taq* polymerase after hybridization. **Gray arrows** indicate the hybridizing reaction. **B:** Mobility shifts of the loop-hybrid bands caused by the mutant allele are shown as the difference (mm) in mobility in the gel between the loop-hybrid band of the mutant allele and that of the normal allele for each LH-G probe, together with the sequence of the loop generated by the LH-G in the sense strand of the normal allele. Partial sequences of the normal and mutant alleles in the sense (s) strand are shown, with the codon L858 in bold. Also shown is the partial reverse anti-sense sequence (a) of the LH-G probe 18R with deleted nucleotides (—). **C:** Paired PCR products from the template DNA of the plasmid clones of the normal (N) and the mutated (m) alleles were examined with a series of LH-G probes as denoted for each lane and analyzed with PAGE. Deletions in these LH-G probes generated the loops of 1 to 18 nucleotide lengths in the loop-hybrids. Control PCR products (—) hybridized with no LH-G probe are also shown.

DNA prepared from fresh lung adenocarcinoma tumor tissues was screened for the L858R mutation using LH-MSA (Figure 2A). The expected double bands for the putatively heterozygous mutation were clearly shown for 3 of 16 examined cases. The L858R mutation in these three cases was confirmed by direct sequencing. For comparison DNA from FFPE tissues of 50 cases in pathological archives of operated lung adenocarcinoma were analyzed by LH-MSA and by direct sequencing. PCR products from those cases in which mutations were detected by LH-MSA were cloned in plasmids and screened with LH-MSA, and mutant clones were sequenced. As summarized in Table 2, 26% of the cases (13 of 50) exhibited the L858R mutation. Nine of these were consistent with the direct sequencing results, but the remaining four were not confirmed because of insufficient quality of the direct

sequence data. One mutation (2%) other than L858R was detected by LH-MSA and determined to be A859T by sequence analysis of the mutant clone and by the direct sequencing. When FFPE tissue DNA from an additional 68 lung adenocarcinoma cases was examined by LH-MSA, the mutation L858R was again observed at a high frequency (27.9%, Table 2). Mutations other than L858R (L861R) were detected at a low frequency (2.9%, 2 of 68) by LH-MSA and sequence analysis of the mutant clones. One of the rare mutations (L861R) produced a mutant loop-hybrid band that shifted differently from that of L858R (Figure 2B). These rare mutations were close in proximity to L858 (Figure 2C). In the present analysis, the mutational state of EGFR exon 21 at L858 was diagnosed by LH-MSA with a high accuracy of 97.5%, taking into account mutations other than L858R (2.5%). Mutations other than

Figure 2. Detection of point mutations at or near the hot spot L858R of EGFR exon 21. **A:** The electropherogram after LH-MSA using LH-G probes 7R and 18R to detect the L858R mutation (CTG>CGG) of EGFR exon 21 in DNA from fresh lung adenocarcinoma tissues. **Lane 1**, the normal allele; **lanes 2 to 4**, putative heterozygous mutations. The homoduplex bands were 161 bp in length. **B:** LH-MSA with probes 7R and 18R to detect the following heterozygous mutations in EGFR exon 21 of L858R (CTG>CGG, **lane 1**), A859T (GCC>ACC, **lane 2**), and L861R (CTG>CGG, **lane 3**). **Lane 4** is the normal allele. **C:** Positions of point mutations in exon 21 detected by LH-MSA (L858R, A859T, L861R; closed triangles). **D:** Detection of the mutated allele (m) of EGFR L858R present at decreasing ratios to the normal allele (N) with LH-MSA using the LH-G probe 18R. The template DNA was constituted by mixing the diluted DNA of cloned plasmids of the mutated and normal alleles. Loop-hybrid bands are shown, together with the observed mutant to normal ratios (m/N) in the PCR products, the standard deviations (SDs), and the total numbers of clones examined (Tn).

L858R that were undetected by LH-MSA could occur, but their presence was not verified in this study.

LH-MSA may be used semiquantitatively, as shown in Figure 2D. The mutant allele in the mixed sample at the mutant to normal ratio 0.05 (0.08 by the observed ratio) was detected with LH-MSA but not detected by dideoxy

Figure 3. Detection of deletion mutations in EGFR exon 19 with LH-MSA. **A:** Schematic representation of loop-hybrids (LH) generated by the normal LH-G probe (Np) hybridized to the anti-sense strands of the PCR products of deleted mutant alleles. Homoduplexes of the deleted mutants are reproduced in LH-MSA by extension of the PCR primer (p) hybridized to the sense strands of the PCR products. The symbols are the same as in Figure 1A. **B:** LH-MSA of PCR products from the plasmid clones of deletion mutant alleles G1 to G7 (**lanes 1 to 7**) and of the normal allele (N) treated with the normal LH-G probe 19JWTF. **C:** Partial nucleotide sequences of the deletion mutants (G1 to G7) and the normal allele (N) of EGFR exon 19, as well as the corresponding normal amino acid sequence. Deleted nucleotides are indicated by dots, and nucleotide displacements are underlined. Incidences of the single deleted mutations in 118 cases of lung adenocarcinoma are shown in parentheses.

sequencing (data not shown). Our results show that LH-MSA was able to detect a mutant allele comprising 7.5% of the total DNA, suggesting that tumor cells with a heterozygous mutation that comprise 15% of the total cell mixture might be detected by LH-MSA.

Detection of In-Frame Deletion Mutations in Lung Adenocarcinoma Using LH-MSA

In-frame deletion mutations in EGFR exon 19 involving 9 to 18 bases (including the overlapping region L747 to E749; Figure 3C) were detected frequently in lung adenocarcinoma.⁵⁻⁹ For heterozygous deletion mutations, heteroduplexes between the normal and the deleted mutant alleles of the PCR product were detected in PAGE as

Table 2. Point Mutations in EGFR Exon 21 Detected by LH-MSA in FFPE Tissue DNA from Lung Adenocarcinomas

Genotype of EGFR exon 21	Experiment 1		Experiment 2	Total
	LH-MSA (%)	Direct sequence (%)	LH-MSA (%)	LH-MSA (%)
Normal	36 (72)	35 (70)	47 (69.1)	83 (70.3)
L858R	13 (26)	9 (18)	19 (27.9)	32 (27.1)
A859T	1 (2)	1 (2)		1 (0.8)
L861R			2 (2.9)	2 (1.7)
Undetermined		5 (10)		
Total	50	50	68	118

Figure 4. Comparison of the mutations in BRAF at V600 and NRAS at Q61 detected by LH-MSA in DNA derived from bulk fresh tumor tissues versus FFPE tumor tissues of thyroid carcinoma. **A:** Loop-hybrid bands after LH-MSA with the LH-G probe 9F for detection of the mutation V600E (GTG>GAG) in BRAF exon 15, using the DNA from fresh tumor papillary thyroid carcinoma tissue. **Lane 1**, the putative heterozygous BRAF mutation; **lane 2**, the normal allele. **B:** Loop-hybrid bands after LH-MSA with the LH-G probe 10K for detection of mutations in NRAS at Q61, using DNA from fresh tumor follicular thyroid carcinoma tissue. **Lanes 1 and 2**, the putative heterozygous NRAS mutations Q61R (CAA>CGA) and Q61K (CAA>AAA), respectively; and **lane 3**, the normal allele. **C:** Loop-hybrid bands for detection of the BRAF mutation V600E, using DNA from FFPE tumor tissues (P) and from bulk fresh tumor tissues (F) of 21 thyroid papillary carcinoma cases. **Asterisks** indicate discrepant results. **D:** Loop-hybrid bands after LH-MSA for detection of the NRAS mutation Q61R in bulk fresh tumor DNA of the discrepant cases with respect to the BRAF mutation. The heterozygous NRAS mutation was evident in cases 21 and 28. **E:** Loop-hybrid bands indicating the heterozygous NRAS mutation Q61R (top) and the heterozygous BRAF mutation V600E (bottom) in DNA derived from follicular-type (fol1, fol2, and fol) and papillary-type (pap) tumors of FFPE tissue sections in the discrepant cases 21 and 28.

a pair of retarded mobility bands easily distinguishable from the homoduplex band showing size-dependent migration (data not shown). LH-MSA was adapted to detect not only heterozygous but also mono-allelic deletion mutations (Figure 3A). Namely, a normal oligonucleotide, 19JWTF, was added as the LH-G probe to the PCR products from plasmid clones of several deleted mutant alleles and hybridized using LH-F steps. Analysis of these reaction products in PAGE revealed various retarded mobility bands of loop-hybrids at specific positions for each of the deleted mutant alleles (Figure 3B). No retarded mobility band was observed for the PCR product of the normal allele treated with this LH-G probe. Using this LH-MSA adapted for the detection of deletion mutations in EGFR exon 19, 49 deletion mutations (41.5%) were detected in DNA samples from FFPE tumor tissues of 118 lung adenocarcinoma cases and these deletions were confirmed by sequencing the cloned mutants. Although most of the cases (45 of 49, 92%) were uniquely associated with one of the deletion mutations shown in Figure 3C, composite mutations of two different in-frame deletions were also found after sequencing mu-

tant clones. They were composed of G1 and G2 (two cases), G1 and G3 (one case), and G1 and G4 (one case) (Figure 3C). These observations implicated multiple mutations in these tumor cases.

Detection of BRAF and NRAS Hot Spot Mutations in Thyroid Carcinoma Using LH-MSA

The kinase-activating mutation V600E in BRAF was shown to occur at high frequencies in papillary thyroid carcinoma,^{19–21} whereas the activating mutation at Q61 in NRAS is prevalent in follicular thyroid carcinoma.^{22,23} LH-MSA was adapted for detection of these mutations. DNA samples prepared from fresh tumor tissues of papillary thyroid tumors were examined by LH-MSA for the V600E BRAF mutation. Distinct double bands (Figure 4A), presumably indicating the heterozygous point mutation, were observed in 64% (16 of 25) of cases, consistent with the previous direct sequencing results. Similarly, DNA samples prepared from fresh tumor tissues of follicular thyroid carcinoma were screened for the NRAS

Figure 5. Different histopathological types of the thyroid tumor in case 28 in tissue sections stained with H&E. **A:** Tumor tissue of the papillary type. **B:** Tumor tissue of the follicular type. Scale bars = 50 μ m.

Q61 mutations using LH-MSA. As shown in Figure 4B, distinct double bands were observed for the putative heterozygous mutations of Q61R and Q61K (26 and 5% of 19 cases, respectively), consistent with predetermined direct sequencing results.

DNA from fresh papillary thyroid carcinoma tumor tissues and from FFPE tumor tissues from the same cases was compared for consistency of the mutations detected by LH-MSA. Of 21 cases compared, 17 yielded the same results using both DNA samples, namely, the BRAF heterozygous V600E mutation in 11 cases and no such mutation in six cases. Discrepant results were observed in four cases (Figure 4C). In one case (no. 11), a single mutant loop-hybrid band was predominant in the FFPE tissue DNA, but heterozygous double bands were observed at equal densities in the fresh tumor DNA. Such differences may be attributed to the loss of heterozygosity in some portions of tumor tissues, including the sampled area of the paraffin section within the tumor heterozygous for the BRAF mutation. The faint shifted loop-hybrid band of the mutant allele in case 25 may suggest that the fresh tumor DNA was contaminated by DNA from normal cells, because equally dense double bands indicating the heterozygous BRAF mutation were detected in the FFPE tumor tissue DNA. In the other two cases (nos. 21 and 28), the normal BRAF in the bulk fresh tumor tissue DNA were discrepant with the apparently heterozygous BRAF mutation in the FFPE tissue DNA. Interestingly, NRAS mutation Q61R was detected in the bulk fresh tumor DNA in these two cases (Figure 4D) but not in the other 19 cases. Histopathological examinations of the tumors documented the presence of both of papillary and follicular tissue types as separate components in the same tumors in these two particular cases, but not in the others (Figure 5, A and B). Tumor DNA from these papillary and follicular tissue types were separately sampled from the FFPE tissue sections and examined for the mutations in BRAF and NRAS using LH-MSA. As shown in Figure 4E, double bands indicating the heterozygous BRAF mutation were detected in the papillary-type but not in the follicular-type tumor tissues. On the other hand, the double bands indicating the heterozygous NRAS mutation were detected in the follicular-type but not in the papillary-type tumor tissues. Because NRAS

mutations tended to be associated with follicular thyroid carcinoma,^{22,23} DNA prepared from fresh bulk tumors before histological diagnosis in the above two thyroid tumor cases seemed to be derived from the follicular-type tumors carrying the mutation in NRAS but not from the papillary-type tumors carrying the mutation in BRAF.

Discussion

LH-MSA is unique in that synthetic oligomers are used for the generation of heteroduplexes bearing small loops by hybridization to PCR products. Differential mobility of the loop-hybrids in native PAGE that reflects a slight change in the nucleotide adjacent to or within the loop was exploited to detect mutational changes present in the DNA. LH-MSA, along with the original UHG technology,^{15–18} may provide a simple and useful system for detection of mutations in various cancers with fixed mutational hot spots. However, detection of widespread loss-of-function mutations in TP53, BRCA1, MLH1, and other cancer-related genes may elude straightforward application of LH-MSA in the present simple format. As shown in this study, LH-MSA is particularly useful for the molecular diagnosis of lung adenocarcinoma because it can easily detect in-frame deletion mutations in exon 19 and the hot spot point mutation L858R in exon 21 of EGFR that is associated with therapeutic responses to EGFR kinase inhibitors such as gefitinib.^{5–9} The V600E point mutation in BRAF could be diagnostically important for thyroid carcinoma^{19–21} and melanoma^{24–26} if specific kinase inhibitor drugs for mutated BRAF become commonly available for therapeutic use.²⁷ LH-MSA was able to detect mutant alleles present at low proportions in the sampled DNA. This might be a valuable feature of LH-MSA for cases in which tumor cells are intermingled with varying amounts of normal cells in available diagnostic samples. Similar sensitivity in the detection of NRAS mutations at low proportions was also achieved using the standard UHG technology.^{17,18}

Point mutations detectable with LH-MSA may not be limited to the genes presently described. Adaptations of LH-MSA to detect various point mutations of the Ras-oncogene KRAS at codons G12 to G13 in various cancers may be pursued. Genotyping of single nucleotide polymorphisms (SNPs) can also theoretically be achieved by LH-MSA at any loci, and adaptation of LH-MSA for various intragenic SNP loci is under investigation. LH-MSA could be incorporated in SNP analyses of risks such as hypersensitivity to certain therapeutic drugs^{28,29} and susceptibility to cancers.³⁰

LH-MSA is applicable to any unique genomic sequences amplified with PCR. LH-G probes to detect point mutations or to genotype SNPs can be designed using a simple hypothesis. Namely, a mismatched base at the site adjacent to the loop in the loop-hybrid will induce a shift in its mobility. Nucleotide lengths of the loop and the positions of the loop relative to the mutational site can be varied to attain optimal shifts of the loop-hybrid band in the presence of the mutation. A faint secondary band that

might confuse interpretation of the results may be virtually eliminated using PAGE-purified LH-G probes. Mutational changes occurring within the loops were detected by LH-MSA as shown for the mutations in EGFR, A859T, and L861R. These instances indicated that there might be some sensitive positions in the loop sequences such that a single nucleotide change therein may affect loop conformation and lead to a mobility shift of the loop-hybrid. In the present study, PAGE was executed at room temperature, and the influence of ambient temperature fluctuation on the retarded mobility of loop-hybrid DNA during electrophoresis appeared to be low. However, detection of mutations within the loops by LH-MSA seemed to be improved by lowering the temperature during electrophoresis (our preliminary observation).

To assess the reliability of LH-MSA for detecting mutations in the DNA of fixed tumor tissues, DNA prepared from fresh tumor tissues and from FFPE tissue sections was compared. Identical results were obtained for the deletion mutations in EGFR exon 19 of lung adenocarcinoma (data not shown). However, a few discrepant results were observed for the point mutation of BRAF in papillary thyroid carcinoma. In two cases, discrepancy was attributed to the presence of two distinct lines of differentiation in thyroid tumor development. The different histopathological types of tumors and their association with distinct mutations in the above two cases may indicate the existence of tumors that were initiated by a common cause but subsequently followed separate courses of tumor development by the mutations of different genes in RAS-RAF signaling pathway. Our detailed analysis not only resolved the discrepancies but also affirmed the tissue-type-specific associations of the BRAF mutation to papillary^{19–21} and the NRAS mutation to follicular^{22,23} thyroid carcinoma. The results for the unusual cases shown above clearly indicated that the DNA derived from FFPE tissues, although low in quality and quantity, could provide precise and important molecular data associated with histopathological diagnosis.

Single LH-bands for each of the mutant and normal alleles were sharp and well separated, and therefore seemed amenable to semiquantitative approaches such as copy number estimation. Simplicity of LH-MSA may allow instantaneous detection of mutations in comparison to other mutation detection systems such as SSCP and UHG, in which four bands derived from normal and mutant strands are usually exhibited. Compared with denaturing high performance liquid chromatography, LH-MSA showed a fourfold higher sensitivity in detection of the L858R mutation in EGFR when serially mixed samples were applied at a low concentration (our preliminary results). The simple LH-MSA detection system may enable rapid and low-cost screening of hot spot mutations in certain types of cancers and genotyping of SNPs in clinical risk assessment. LH-MSA and silver staining of the gel after PAGE may facilitate genetic examinations in minimal laboratory settings. Use of LH-MSA for genotyping analysis may not be limited to humans but might be extended to any organism. Current genome-wide searches for SNP loci associated with specific phenotypes and for mutations responsible for hereditary dis-

eases in humans and domestic animals may be greatly facilitated by simple genotyping tools such as LH-MSA.

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