Symposium article

Novel P53 mutations detected by FAMA in colorectal cancers

F. De Galitiis[1], K. Cannita[1], A. Tessitore[1], F. Martella[1], Z. C. Di Rocco[1], A. Russo[2], V. Adamo[3], S. Iacobelli[4], S. Martinotti[5], P. Marchetti[1], C. Ficorella[1] & E. Ricevuto[1]*
On behalf of CINBO (Consorzio Interuniversitario Nazionale Bio-Oncologia)

[1]U.O. Medical Oncology, San Salvatore Hospital, University of L'Aquila; [2]Department of Surgery and Oncology, Medical Oncology Section, Universitàdi Palermo; [3]U.O. Medical Oncology, University of Messina; [4]U.O. Medical Oncology, University of Chieti; [5]U.O. Clinical Pathology, University of Chieti, Italy

Background: The aim of the study was to identify p53 gene mutations by FAMA (fluorescence-assisted mismatch analysis) in colorectal cancers.

Patients and methods: Analytical scanning of the p53 gene (exons 5–9) was performed in colon cancer samples from 44 consecutive patients by FAMA. FAMA is a semiautomatic scanning approach based on the chemical cleavage of the mismatch in fluorescently labeled heteroduplex DNA, obtained from the combination of a normal and a mutated allele. FAMA has already shown optimal levels of diagnostic accuracy and sensitivity in detecting gene mutations (nucleotide substitutions, insertions/deletions) both at the germline and somatic level. The peculiar feature of FAMA is its ability to detect and localize mutations, by a redundant pattern of signals due to fluorescent DNA fragments generated by chemical cleavage. Moreover, previous data have demonstrated that normal contaminating DNA from stromal cells in the sample does not affect the sensitivity of the procedure, leading to the identification of the mutation even when the ratio mutant/normal allele is 10%.

Results: Eighteen mutations (12 missense, one nonsense, two deletions, three nucleotide substitutions at the level of the splice-junctions) and two polymorphisms were detected by FAMA in 17 patients (39%) and then confirmed by automated sequence analysis. Six of 18 mutations (33%) were not previously reported for colon cancer samples and two of 18 lesions (11%) were identified as novel p53 mutations.

Conclusions: Analytical scanning of the p53 gene by FAMA in DNA from colon cancer samples provides a sensitive, accurate and specific diagnostic procedure for routine clinical application.

Key words: colon cancer, p53, mutations, FAMA

introduction

The occurrence of the inactivation of the p53 tumor suppressor gene (located in the short arm of chromosome 17 and encoding for a 53-kD nuclear phosphoprotein) is part of the chromosomal instability characterizing sporadic cancers and represents a specific, wide, frequently detected, recurring genetic event in solid tumors and also a predictive and prognostic indicator at the clinical level [1–3]. The p53 gene plays a central role in multiple cellular pathways, being implicated in the cell cycle control, DNA repair, cell differentiation and programmed cell death [4–8].

Mutations in the p53 tumor suppressor gene are identified in approximately 35%–45% [9–12] of colorectal cancers, and it may be associated with worse prognosis and chemo/radioresistance [13, 14]. Most of the colon cancer patients present a B2-C pathologic stage and to date a small but significant clinical benefit of adjuvant chemotherapy has been

demonstrated in Duke's C colon cancer, while it is still debated in Duke's B2 [15–17]. Thus, the identification of prognostic and/or predictive molecular parameters, such as p53, represents one of the most important aims [18–23].

Controversial data exist about clinical implications of p53 alterations, due to the different levels of accuracy of the diagnostic procedures [24–29].The alterations of the p53 gene are usually detected by scanning procedures reaching different levels of accuracy (SSCP, DGGE, DHPLC, CCM) and/or automated sequencing. The contamination of tumor sample by genetically normal stromal cells (fibroblasts, endothelial cells, lymphocytes) represents a very important factor in limiting the accuracy of diagnostic methods at the somatic level [29] and it constitutes a critical factor when the molecular diagnosis is applied in clinical contexts. In this regard, previous data have demonstrated that most of the diagnostic techniques are not sensitive when the contaminating normal DNA is more than 20% of the total content of the sample. In particular, direct sequencing fails to identify a mutation (nucleotide substitution) when more than 16% of normal contaminating DNA is present in the sample [27, 30].

*Correspondence to: Dr E. Ricevuto, Università degli Studi di L'Aquila, Dipartimento di Medicina Sperimentale, Via Vetoio, Coppito II 67100, L'Aquila, Italia. Tel: +39-0862-433582; Fax: +39-0862-433523; E-mail: enrico.ricevuto@univaq.it

FAMA is a semiautomatic scanning approach based on the chemical cleavage of the mismatch in fluorescently labeled heteroduplex DNA, obtained from matching a normal and a mutated alleles. The normal DNA drives all mutant DNA into hybridization, thus permitting the heteroduplexes formation. The subsequent chemical cleavage of the mismatch and the formation of shorter fluorescent DNA extra fragments define the mutation. Thus, the FAMA diagnostic approach is not affected by normal DNA contamination, which is usually present when a molecular diagnosis at the somatic level is performed, but, on the contrary, it takes advantage and fails to detect mutation at normal allelic rate ≤8%. In addition, a peculiar feature of FAMA is the ability to detect, precisely locate and define genetic mutations, even if multiple and different, along the same fluorescent DNA fragment up to 1.3 kb

in length [30, 31]. Therefore, molecular scanning of p53 gene by FAMA represents one of the most accurate and sensitive diagnostic procedures. In this study, FAMA was performed in 44 tumor samples in order to evaluate better the spectrum of p53 mutations in colorectal cancer.

patients and methods

samples and DNA extraction

Forty-four samples of primary colorectal cancer from surgically treated patients followed at the San Salvatore Hospital of L'Aquila were consecutively collected and then analyzed for p53 gene mutations. Tumor samples were stored at −80°C at the time of surgery. After histologic confirmation of colorectal carcinoma, genomic DNA was extracted,
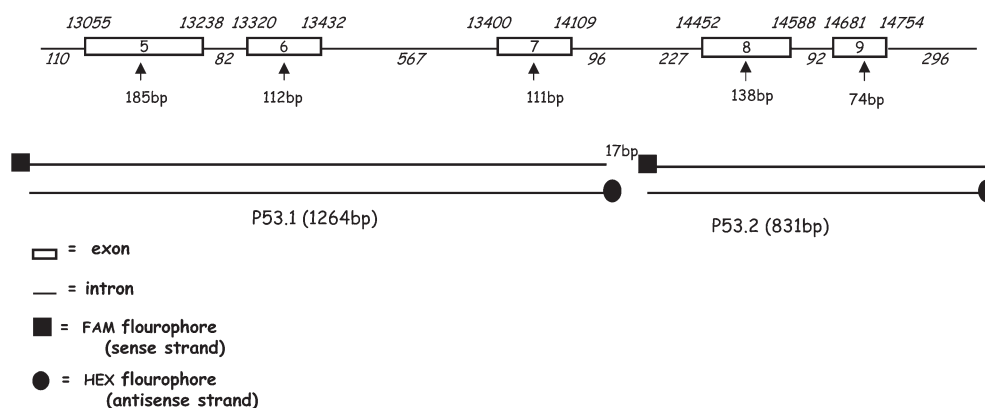

Figure 1. p53 gene amplicons for F.A.M.A.

Table 1. p53 mutations in colon cancer by FAMA

Samples	Exon	Nu. Change	AA change	Type	IARC database	
					Colon	Other cancers
C94	5	GCC 13065 GGC	Ala 129 Gly	Missense	Not reported	Not reported
C17	5	CAA13094 CCA	Gln 136 Pro	Missense	Not reported	Reported
C1	5	GAG 13189 CAG	Glu 171 Gln	Missense	Reported	Reported
C118	5	CGC 13203 CAC	Arg 175 His	Missense	Reported	Reported
C125	5	CGC 13203 CAC	Arg 175 His	Missense	Reported	Reported
C122	5	TGC 13207 TGG	Cys 176 Trp	Missense	Not reported	Reported
C36	6	CAG 13334 TAG	Arg 192 Stop	Nonsense	Reported	Reported
C38	6	13370 del G	Val 204	Deletion	Not reported	Reported
	S.j.	AG gtc 13434 AG gcc		Splice junction	Reported	Reported
	int 6	cgc 13965 ccc		Polymorphism		
C5	S.j.	AG gtc 13434 AG gcc		Splice junction	Reported	Reported
C106	S.j.	AG gtc 13434 AG gcc		Splice junction	Reported	Reported
C22	7	14030 del A	Asn 235	Deltion	Not reported	Not reported
C85	7	GGC 14060 AGC	Gly 245 Ser	Missense	Reported	Reported
C112	7	CGG 14070 CTG	Arg 248 Leu	Missense	Reported	Reported
C70	7	ATC 14069 ACC	Ile 247 Thr	Missense	Not reported	Reported
C30	8	CTG 14463 CCG	Leu 265 Pro	Missense	Reported	Reported
C39	8	CGG 14513 TGG	Arg 282 Trp	Missense	Reported	Reported
C127	8	CGG 14513 TGG	Arg 282 Trp	Missense	Reported	Reported

Table 2. Mutation features

	N	%	IARC database %	Novel mutations
Samples	44			
Wild-type	27	61		
Mutant	17	39	43.6	
Mutation type				
Missense	12	67	73	3
Nonsense	1	5	7	
Splice junction	3	17	2	
Deletion	2	11	9	2
Substitutions				
A:T>C:G	1	5	4	
A:T>G:C	3	16	11	
A:T>T:A	0	0	5	
G:C>A:T	6	36	44	
G:C>C:G	3	16	7	
G:C>T:A	1	5	15	
Exon mutation distribution				
Exon 5	6	33	33	2
Exon 6	2	11	8	1
Exon 7	4	22	29	2
Exon 8	3	17	25	0
Exon 9	0	0		
Splice junction	3	17		
Hot spot				
175	2	11	14	
245	1	5	6	
248	1	5	13	
273	0	0	7.5	
282	2	11	6	
Total	6	35	23	

according to the proteinase K digestion and phenol/chloroform extraction protocol.

detection of p53 gene mutations

Analytical scanning of the p53 core domain (exons 5–9) was planned by FAMA, a semiautomatic scanning procedure based on the chemical cleavage of mismatch of heteroduplex DNA molecules, which has demonstrated optimal diagnostic accuracy even for large PCR fluorescent amplicons (up to 1.3 kb in length) [31, 32]. P53 core domain was analyzed using two fluorescent amplicons (Figure 1), as previously reported by our group [30]: p53.1, 1264 bp, spanning exons 5–7 and adjacent introns and p53.2, 831 bp, spanning exons 8–9 and flanking introns. Fluorescent primer pairs conjugated at the 5′-end with FAM (sense strand) and HEX (antisense strand) fluorophores were used [30]. All primers were selected within intronic DNA sequence, at least 100 nucleotides apart from exons, and contained a 'GG' dinucleotide at the 5′-end as a spacer between the fluorophore and the DNA sequence: p53.1 forward /5′-FAM-GGTTGCAGGAGGTGCTTACA-3′; p53.1 reverse /5′-HEX-GGTATGGAAGAAATCGGTAAGA-3′; p53.2 forward /5′-6-FAM-GGTCATCACATTTCCGGCGG-3′; p53.2 reverse /5′-HEX-GGAAGTAACTCCATCGTAAGTC-3′. PCR reactions (35 cycles: 30′, 94°C; 30′, 56°C; 1.20′, 72°C) were performed using 200 ng of genomic DNA; 10 pmoles of fluorescent primers, $1 \times$ PCR buffer (50 mM Tris pH 9.2, 16 mM $(NH_4)_2SO_4$, 2.25 mM $MgCl_2$), Taq DNA polymerase 1.25 IU, in a total volume of 25 µl. Then heteroduplex formation and chemical

cleavage reactions were carried out as previously described [31, 32]. The electrophoresis was performed on an Applied Biosystems ABI PRISM 377 DNA sequencer. The electrophoresis results were analyzed using the GeneScan 3.1 software. All mutations observed by FAMA were confirmed by direct sequencing, using the Big Dye Terminator Ready Reaction premix (Applied Biosystems) according to the manufacturer's instructions. Each sample was amplified and 50 ng were then purified and used for sequencing reactions, using both upstream and downstream primers. The following primer pairs, specific for exons 5, 6, 7 and 8–9 were used: p53.1 forward, p53.1 reverse, p53.2 forward and p53.2 reverse as previously described, but not containing the fluorophore at the 5′ end; and p53.5 reverse, p53.6 forward, p53.6 reverse, p53.7 forward as already described. In some case, mono-allelic sequencing was performed by subcloning single alleles using 'pGEM-T easy vector' (Promega, madison, WI), according to the manufacturer's instructions.

results

Analytical scanning by FAMA of the p53 gene (exons 5–9) was performed in 44 colorectal cancer samples. Table 1 describes the 18 p53 mutations detected in 17 patients (39%), one patient showed a double p53 gene mutation. The mutation distribution in the exons subjected to analysis was: exon 5, 6 (33%); exon 6, 2 (11%); exon 6 splice-site, 3 (17%); exon 7, 4 (22%); exon 8, 3 (17%). Twelve missense (67%), one nonsense (5%), three splice site mutations (17%) and two microdeletions (11%) generating an early stop codon were found. Six mutations (35%) were ascribed to hot spot regions: codon 175, 2; codon 245, 1; codon 248, 1; codon 282, 2. The splice site mutation was observed in three samples. Overall, 14 different mutations were observed. Two novel p53 mutations were identified in this study: exon 5, C13065G (Ala 129 Gly); exon 7, 14030 delA, generating an early stop codon (Table 2). Six of the identified mutations were never reported in colon cancer samples.

A missense mutation (C122 sample) never reported in colorectal cancer (C13207G, Lys 176 Trp) is shown in Figure 2. Extra bands are displayed in the gel file (Figure 2A) due to fluorescent heteroduplex DNAs subjected to chemical cleavage that are also identified by peaks in the electropherogram's plots (Figure 2B, C), specifically defining the position and the nature of the mutation. The two extra bands in the electrophoretic lane of the sample treated with hydroxylamine indicate a couple of differentially labeled DNA fragments obtained by the chemical cleavage of sense (261 nt, blue, marked with FAM) and antisense (1003 nt, green, marked with HEX) strands, which represent the specific signature of a C/G substitution.

Interestingly, the samples C38 (Figure 3) showed a couple of extra bands, characterizing the specific signature of two different mutations as well as an intronic polymorphic variant in the same p53.1 amplicon. The extra fragments marked with FAM (blue, sense strand) fluorophore on HA and OT lane (Figure 3A, B, D), 491 nt in length, are due to a T>C nucleotide substitution at the level of the donor splice junction of exon 6 (Figure 3A, C, D). The extra fragments in the gel file (Figure 3A) marked with FAM (blue, sense strand) on the OT lane and with HEX (green, antisense strand) on HA lane, each 421 nt and 843 nt in length (Figure 3A, C, D), respectively, describe a 13370 delG mutation within the exon 6, as confirmed by mono-allelic sequencing (Figure 3F). Moreover, the extra fragments in the HA lane,
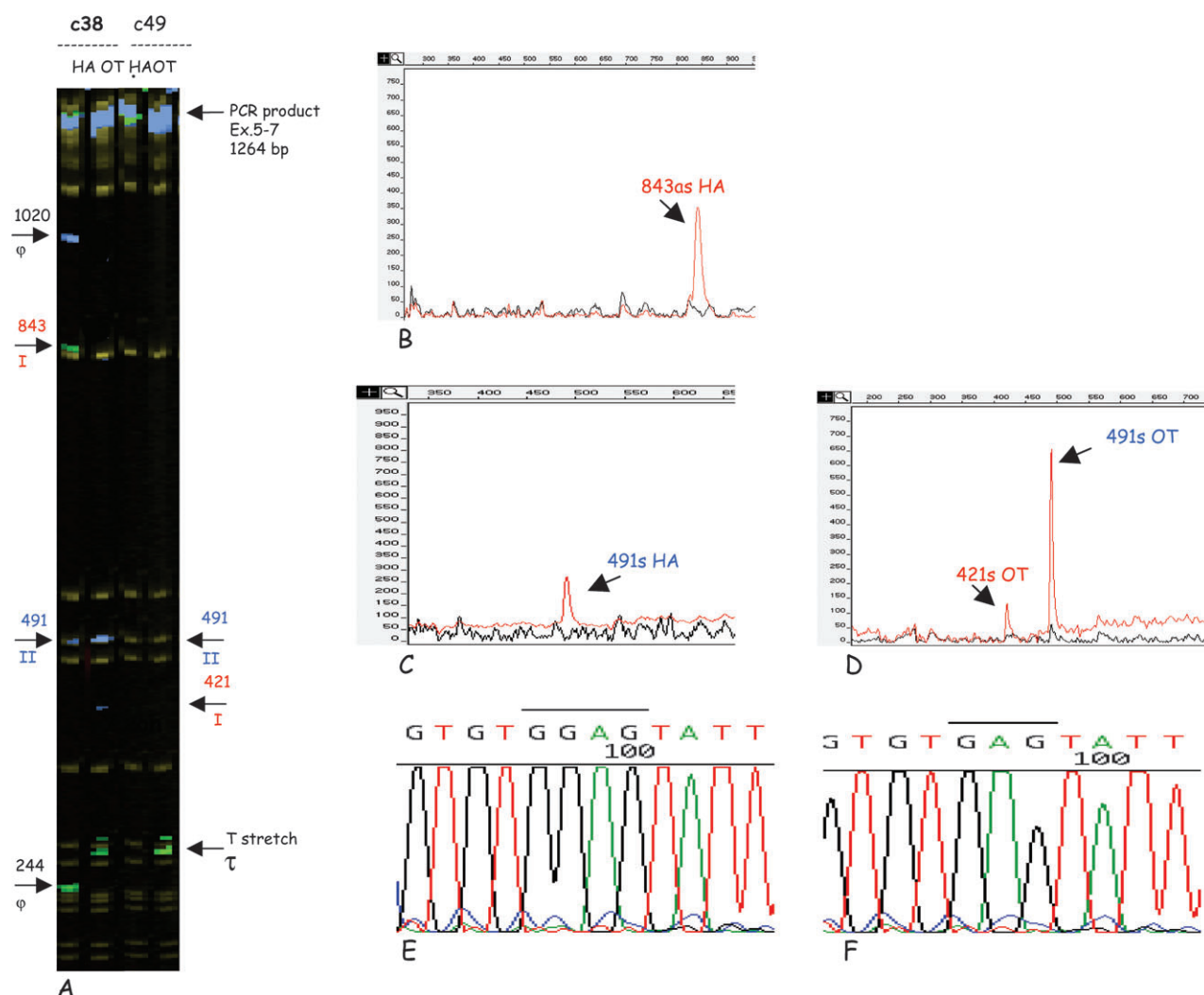
Figure 2. Sample C38. (**A**) Gel imageof samples C38 and C49 (control) : in black polimorphism and T stretch FAMA signal; in red mutation at codon 204 (13370 del G) FAMA signal; in blue mutation at splice site (AG gtc 13434 AG gcc) FAMA signal (**B**) F.AM.A. Electropherogram of sample treated with HA (sense strand); (**C**) F.AM.A. Electropherogram of sample treated with HA (antisense strand); (**D**) F.AM.A. Electropherogram of sample treated with OT (sense strand); (**E**) F.AM.A. Electropherogram of sample treated with OT (antisense strand); F Automated mono-allelic sequencing of a wt allele; G Automated mono-allelic sequencing of the mutans allele.

marked with FAM and HEX fluorophores, respectively 1020 and 244 nt in length (Figure 2A), describe a polymorphic variant within the intron 6 (cgc 13965 ccc). The last two sequence variants were also confirmed by automated sequencing (Figure 3).

discussion

The analytical scanning of the p53 gene performed by FAMA in 44 colorectal cancer DNA samples confirms that FAMA is a feasible procedure for molecular diagnosis of both germline and somatic mutations [32, 33]. The redundancy of signals obtained by the chemical cleavage, even in the presence of a low mutant/normal allele ratio (around 10%), makes it suitable for identifying exactly both the nature and the position of the genetic lesion. The p53 core domain was examined by scanning only two long fluorescent amplicons containing exons 5–9 and flanking introns. The rate of mutation was 39% (17/44),

according to that reported in the IARC database R10 (43%, range 5%–100%; CI 31% to 55%, median p53 mutations), which shows all known p53 mutations identified with different diagnostic procedures. In addition, the rate of the different types of mutations detected in this study was equivalent to that reported in the IARC database (missense 66.6% versus 73% IARC, deletions 11% versus 9% IARC, nonsense 5% versus 7% IARC). A stronger incidence of splice-junction mutations was detected (17% versus 2%), most probably due to the diagnostic strategy as well as to the primers selected far from the exon splice-site. Interestingly, two novel 53 mutations were identified: one deletion (exon 7, 14030 delA, generating an early stop codon) and one missense (exon 5, C13065G; Ala 129 Gly) unreported in the IARC database (last updated July 2005).

Overall, FAMA detected two unreported mutations in all type of cancer (2/14; 14%) and six unreported mutations in colon cancer (6/14; 43%). In conclusion, the analysis by FAMA of
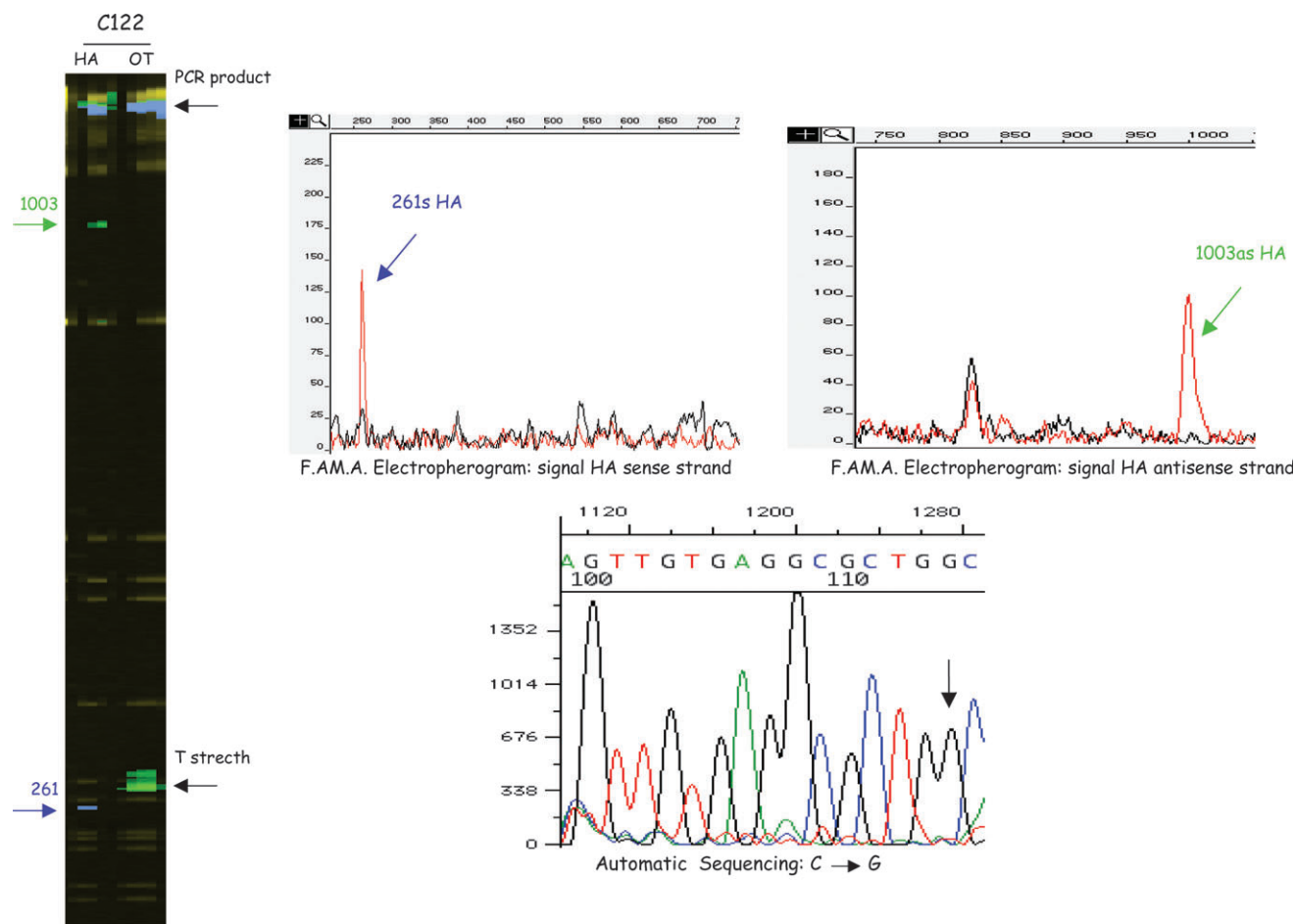
Figure 3. Sample C122.

colon cancer DNA samples has confirmed its diagnostic accuracy at the somatic level, even in detecting multiple sequence variants in the same amplicon, with consequent time reduction of the molecular analysis.

references

1. Levine AJ, Momand J, Finlay CA. The p53 tumour suppressor gene. Nature 1991; 351: 453–456.

2. Vogelstein B, Fearon ER, Hamilton SR et al. Genetic alterations during colorectal-tumor development. N Engl J Med 1988; 319: 525–532.

3. Donehower LA. Effects of p53 mutation on tumor progression: recent insights from mouse tumor models. Biochim Biophys Acta 1996; 1242: 171–176.

4. Diller L, Kassel J, Nelson CE et al. p53 functions as a cell cycle control protein in osteosarcomas. Mol Cell Biol 1990; 10: 5772–5781.

5. Lowe SW, Ruley HE, Jacks T, Housman DE. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell 1993; 74: 957–967.

6. Kastan MB, Onyekwere O, Sidransky D, Vogelstein B, Craig RW. Participation of p53 protein in the cellular response to DNA damage. Cancer Res 1991; 51: 6304–6311.

7. Livingstone LR, White A, Sprouse J et al. Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53. Cell 1992; 70: 923–935.

8. Kern SE, Kinzler KW, Bruskin A et al. Identification of p53 as a sequence-specific DNA-binding protein. Science 1991; 252: 1708–1711.

9. Samowitz WS, Curtin K, Ma KN et al. Prognostic significance of p53 mutations in colon cancer at the population level. Int J Cancer 2002; 99: 597–602.

10. Tortola S, Marcuello E, Gonzalez I et al. p53 and K-ras gene mutations correlate with tumor aggressiveness but are not of routine prognostic value in colorectal cancer. J Clin Oncol 1999; 17: 1375–1381.

11. Hernandez-Boussard T, Rodriguez-Tome P, Montesano R, Hainaut P. IARC p53 mutation database: a relational database to compile and analyze p53 mutations in human tumors and cell lines. International Agency for Research on Cancer. Hum Mutat 1999; 14: 1–8.

12. Soong R, Powell B, Elsaleh H et al. Prognostic significance of TP53 gene mutation in 995 cases of colorectal carcinoma. Influence of tumour site, stage, adjuvant chemotherapy and type of mutation. Eur J Cancer 2000; 36: 2053–2060.

13. Lowe SW, Schmitt EM, Smith SW, Osborne BA, Jacks T. p53 is required for radiation-induced apoptosis in mouse thymocytes. Nature 1993; 362: 847–849.

14. Lowe SW, Bodis S, McClatchey A et al. p53 status and the efficacy of cancer therapy in vivo. Science 1994; 266: 807–810.

15. Allegra CJ, Paik S, Colangelo LH et al. Prognostic value of thymidylate synthase, Ki-67, and p53 in patients with Dukes' B and C colon cancer: a National Cancer Institute-National Surgical Adjuvant Breast and Bowel Project collaborative study. J Clin Oncol 2003; 21: 241–250.

16. Smith RE, Colangelo L, Wieand HS, Begovic M, Wolmark N. Randomized trial of adjuvant therapy in colon carcinoma: 10-year results of NSABP protocol C-01. J Natl Cancer Inst 2004; 96: 1128–1132.

17. Wolmark N, Colangelo L, Wieand S. National Surgical Adjuvant Breast and Bowel Project trials in colon cancer. Semin Oncol 2001; 28 (1 Suppl 1): 9–13.

18. Graziano F, Cascinu S. Prognostic molecular markers for planning adjuvant chemotherapy trials in Dukes' B colorectal cancer patients: how much evidence is enough? Ann Oncol 2003; 14: 1026–1038.

19. Elsaleh H, Powell B, Soontrapornchai P et al. p53 gene mutation, microsatellite instability and adjuvant chemotherapy: impact on survival of 388 patients with Dukes' C colon carcinoma. Oncology 2000; 58: 52–59.

20. Allegra CJ, Paik S, Colangelo LH et al. Prognostic value of thymidylate synthase, Ki-67, and p53 in patients with Dukes' B and C colon cancer: a National Cancer Institute-National Surgical Adjuvant Breast and Bowel Project collaborative study. J Clin Oncol 2003; 21: 241–250.

21. Allegra CJ, Parr AL, Wold LE et al. Investigation of the prognostic and predictive value of thymidylate synthase, p53, and Ki-67 in patients with locally advanced colon cancer. J Clin Oncol 2002; 20: 1735–1743.

22. Iacopetta B, Russo A, Bazan V et al. Functional categories of TP53 mutation in colorectal cancer: results of an International Collaborative Study. Ann Oncol 2006; Mar 8.

23. Russo A, Bazan V, Iacopetta B et al. TP53-CRC Collaborative Study Group. The TP53 colorectal cancer international collaborative study on the prognostic and predictive significance of p53 mutation: influence of tumor site, type of mutation, and adjuvant treatment. J Clin Oncol 2005; 23: 7518–7528.

24. Soong R, Iacopetta BJ, Harvey JM et al. Detection of p53 gene mutation by rapid PCR-SSCP and its association with poor survival in breast cancer. Int J Cancer 1997; 74: 642–647.

25. Soong R, Robbins PD, Dix BR et al. Concordance between p53 protein overexpression and gene mutation in a large series of common human carcinomas. Hum Pathol 1996; 27: 1050–1055.

26. Calzolari A, Chiarelli I, Bianchi S et al. Immunohistochemical vs. molecular biology methods. Complementary techniques for effective screening of p53 alterations in head and neck cancer. Am J Clin Pathol 1997; 107:7–11.

27. Kressner U, Inganas M, Byding S et al. Prognostic value of p53 genetic changes in colorectal cancer. J Clin Oncol 1999; 17: 593–599.

28. Kressner U, Lindmark G, Gerdin B, Pahlman L, Glimelius B. Immunohistological p53 staining is of limited value in the staging and prognostic prediction of colorectal cancer. Anticancer Res 1996; 16: 951–957.

29. Soong R, Iacopetta BJ, Harvey JM et al. Detection of p53 gene mutation by rapid PCR-SSCP and its association with poor survival in breast cancer. Int J Cancer 1997; 74: 642–647.

30. Tessitore A, Di Rocco ZC, Cannita K et al. High sensitivity of detection of TP53 somatic mutations by fluorescence-assisted mismatch analysis. Genes Chromosomes Cancer 2002; 35: 86–91.

31. Ricevuto E, Sobol H, Stoppa-Lyonnet D et al. Diagnostic strategy for analytical scanning of BRCA1 gene by fluorescence-assisted mismatch analysis using large, bifluorescently labeled amplicons. Clin Cancer Res 2001; 7: 1638–1646.

32. Verpy E, Biasotto M, Meo T, Tosi M. Efficient detection of point mutations on color-coded strands of target DNA. Proc Natl Acad Sci USA 1994; 91: 1873–1877.

33. Ricevuto E, Ficorella C, Fusco C et al. Molecular diagnosis of p53 mutations in gastric carcinoma by touch preparation. Am J Pathol 1996; 148: 405–413.