Next-generation sequencing analysis of lung and colon carcinomas reveals a variety of genetic alterations

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Abstract. The development of targeted therapies in cancer has accelerated the development of molecular diagnosis. This new cancer discipline is booming, with an increasing number of gene alterations to analyze in a growing number of patients. To deal with this fast-developing activity, current analysis techniques (Sanger sequencing, allelic discrimination and high resolution melting) take more and more time. In recent years, next generation sequencing (NGS) technologies have appeared and given new perspectives in oncology. In this study, we analyzed FFPE lung and colon carcinomas using the Truseq Cancer Panel, which analyzes the mutation hotspots of 48 genes. We also tested the use of whole-genome amplification before NGS analysis. NGS results were compared with the data obtained from routine diagnosis. All of the alterations routinely observed were identified by NGS. Moreover, NGS revealed mutations in the *KRAS* and *EGFR* genes in patients diagnosed as wild-type by routine techniques. NGS also identified concomitant mutations in *EGFR* and *KRAS* or *BRAF* mutations, and a 15-nt deletion in exon 19 of *EGFR* in colon carcinomas. The study of the other genes sequenced in the Panel revealed 14 genes altered by 27 different mutations and three SNP with a possible role in cancer susceptibility or in the response to treatment. In conclusion, this study showed that NGS analysis could be used for the analysis of gDNA extracted from FFPE tissues. However, given the high sensitivity of this technology, high-throughput clinical trials are needed to confirm its reliability for the molecular diagnosis of cancer.

Introduction

Lung and colon cancers are among the main causes of death in developed countries. The life expectancy of patients is very limited, especially in metastatic disease. Nevertheless, in recent years, the development of targeted therapies (Tyrosine Kinase inhibitors or inhibitors of receptors which hyperactivate survival pathways) has shown great therapeutic promise. For example, patients with a mutated *EGFR* gene and wild-type *KRAS* gene lung tumor are eligible for gefitinib therapy (1). In colon cancer, patients with wild-type *KRAS* and *BRAF* could be treated with panitumumab or cetuximab (2). In skin cancer, vemurafenib (3) or imatinib (4) can be used to treat mutated *BRAF* (V600E) or *c-KIT* melanoma, respectively. As the efficacy of these targeted therapies depends on specific genetic abnormalities, molecular diagnosis has become essential for the treatment of cancers. Since 2008, molecular biology platforms have screened for genetic alterations in *EGFR* (exons 18-21), *KRAS* (codons 12 and 13) and *BRAF* (codon 600). At the beginning, the gold standard was Sanger sequencing, but the technique has low sensitivity and is expensive. With the increasing number of samples and gene alterations to screen for, alternon-amplified techniques were developed. For example, allelic discrimination (5) was developed to screen for a specific mutation quickly at a relatively low price. In parallel, screening technologies, such as High Resolution Melting (6) or fragment analysis (for indel alterations), were developed to use with Sanger sequencing, but only in the presence of potential mutations in the region of interest. Over the years, the number of genes with genetic alterations that could be targeted by therapies has increased rapidly. This medical progress has led to the need for more and more molecular diagnoses. This need has now been met by the recent development of Next Generation Sequencing (NGS), which has revolutionized molecular diagnosis. Indeed, the sequencing capacity allows the analysis of dozens of genes on multiplexed samples. In this paper, we describe the results we obtained with the Truseq Cancer Panel. In addition to the routinely detected mutations, NGS analysis, thanks to its high sensitivity, revealed new mutations in routinely analyzed genes.

Materials and methods

Patients and DNA samples. Eighteen tissue samples with >400 ng gDNA (Table I) from patients treated at the Centre Georges-François Leclerc between 2009 and 2013 were randomly chosen. Genomic DNA was extracted from FFPE tissues

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Table I. Clinical details of studied patients.

Patients	Organ of origin	Histology	Age (years)
L1	Lung	Keratinizing poorly differentiated squamous carcinoma	61
L2	Lung	Moderately differentiated adenocarcinoma	67
L3	Lung	Poorly differentiated adenocarcinoma	62
L4	Lung	Adenocarcinoma	50
L5	Lung	Adenocarcinoma	69
L6	Lung	Adenocarcinoma	55
L7	Lung	Mucus-secreting adenocarcinoma	68
L8	Lung	Acinar differentiated mucus-secreting adenocarcinoma	86
C1	Colon	Moderately differentiated adenocarcinoma	55
C2	Colon	Adenocarcinoma	64
C3	Colon	Moderately differentiated adenocarcinoma	59
C4	Colon	Adenocarcinoma	72
C5	Colon	Adenosquamous adenocarcinoma	70
C6	Colon	Poorly differentiated adenocarcinoma	55
C7	Colon	Adenocarcinoma	80
C8	Colon	Poorly differentiated adenocarcinoma	72
C9	Colon	Well differentiated infiltrating lieberkunien adenocarcinoma	58
C10	Colon	Colloidal adenocarcinoma	67

with either the QIAamp DNA mini kit (Qiagen, Heidelberg, Germany) or the Maxwell 16 FFPE Plus LEV DNA purification kit (Promega, Madison, USA). The samples had already been genotyped by allelic discrimination, fragment analysis and Sanger sequencing. Written consent was provided by all patients, and the researchers obtained authorization from the diagnostic centers to use the tumor samples.

Whole genome amplification. The Repli-g FFPE kit (Qiagen) was used to amplify 300 ng of gDNA from patients L7 and L8: 10 μl of gDNA solution were mixed with 8 μl of FFPE buffer, 1 μl of ligation enzyme and 1 μl of FFPE enzyme. The solution was then incubated at 24˚C for 30 min, at 95˚C for 5 min and then kept at 4˚C. The Repli-g master mix was prepared by mixing, per sample, 29 μl of Repli-g Midi reaction buffer and 1 μl of Repli-g Midi DNA Polymerase. This second mixture (30 μl) was added to the gDNA solution. This solution was then incubated at 30˚C for 2 h, 95˚C for 10 min and kept at 4˚C. Amplified DNA was stored at -20˚C. Thanks to this protocol, we obtained 6900 and 6400 ng of amplified gDNA.

Preparation of libraries. Libraries were prepared with the Truseq Cancer Panel (Illumina, San Diego, USA) by following the manufacturer protocol. Briefly, 400-1250 ng of gDNA in 5 μl water was hybridized with an oligo pool. Then, unbound oligos were removed, and extension-ligation of bound oligos was followed by PCR amplification. PCR products were cleaned and checked for quality using Tapestation analysis (Agilent). The PCR product size had to be around 350 bp. Before sequencing, the libraries were normalized by the normalization process of the Truseq Cancer Panel.

Sequencing with MiSeq device. As each library possessed a specific primer index combination (i5 and i7), the libraries were pooled for 2 sequencing runs (pool no. 1, 10 libraries; pool no. 2, 9 libraries). For the MiSeq sample sheet, each sample was identified by its specific index combination. Libraries were paired-end sequenced with 2x151 bp cycles.

Analysis of obtained sequences. At the end of the run, sequences were aligned to the human genome reference hg19. Generated BAM files were analyzed with the Genome Golden Helix software (Golden Helix, Bozeman, USA). A genetic variation was defined by a Q-score above 30 (except for indel alteration).

Results

All mutations detected with standard methods were detected with NGS. In the routine diagnosis of lung or colon carcinomas, mutations in *KRAS* (exon 2), *EGFR* (exon 18-21), *BRAF* (exon 15) and *HER2* (exon 20) genes are analyzed using three different methods: allelic discrimination for targeted mutations, fragment analysis for the screening of indel variations and Sanger sequencing for non-targeted mutations or characterization of indel abnormalities detected by fragment analysis. All mutation hotspots are analyzed one by one. Over the years, more and more genes and mutation hotspots will need to be explored. For example, exons 3 and 4 of *KRAS*, and exons 2-4 of *NRAS* and *HRAS* need to be analyzed before anti-EGFR antibody can be prescribed for colon cancer (7,8). In the first step of our study, we used the Truseq Cancer Panel kit to sequence samples that had already been analyzed in routine diagnosis. We then compared the results obtained by NGS with the results of the routine

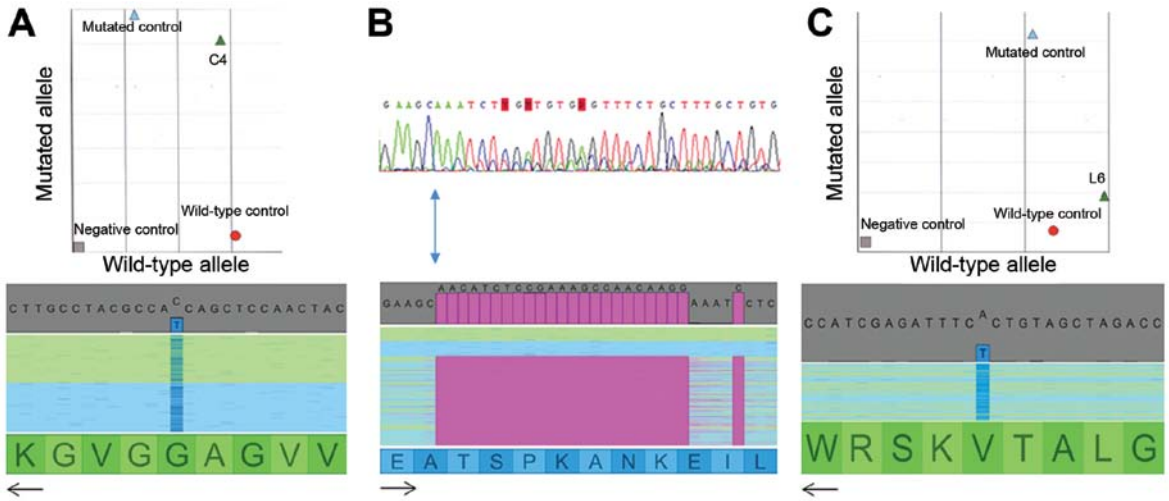

Figure 1. All the mutations detected during routine diagnosis were also detected by NGS. (A) G12D *KRAS* mutation detected by allelic discrimination (upper panel) and by NGS (lower panel) for patient C4. Orientation of the *KRAS* gene needs to be completed to obtain the coding sequence. (B) A 24-nt deletion in exon 19 of *EGFR* obtained with Sanger sequencing (upper panel) for patient L3. Unlike the Sanger method, NGS analysis (lower panel), made it easy to identify the exact deletion (23 nt + 1 nt). The double blue arrow shows the start of the deletion. (C) V600E *BRAF* mutation detected by allelic discrimination (upper panel) and by NGS (lower panel) for patient L6. Orientation of the *BRAF* gene needs to be completed to obtain the coding sequence. The arrows indicate the orientation of each gene.

Table II. Comparison of results obtained routinely and with NGS.

	KRAS		*BRAF*		*EGFR*		*HER2*	
Patients	Routine[a]	NGS	Routine[b]	NGS	Routine[c]	NGS	Routine[d]	NGS
L1	**WT**	**G12D**	WT	WT	**WT**	**15-nt E19**	WT	WT
L2	WT	WT	WT	WT	15-nt E19	15-nt E19	WT	WT
L3	WT	WT	WT	WT	24-nt E19	24-nt E19	WT	WT
L4	WT	WT	WT	WT	L858R	L858R	WT	WT
L5	WT	WT	WT	WT	15-nt E19 T790M	15-nt E19 T790M	WT	WT
L6	WT	WT	V600E	V600E	**WT**	**15-nt E19**	WT	WT
L7	G12C	G12C	WT	WT	WT	WT	WT	WT
L8	WT	WT	WT	WT	WT	WT	WT	WT
C1	WT	WT	**WT**	**V600R**	ND		ND	
C2	WT	WT	WT	WT	ND		ND	
C3	G13D	G13D	WT	WT	ND		ND	
C4	G12D	G12D	WT	WT	ND		ND	
C5	WT	WT	WT	WT	ND		ND	
C6	WT	WT	WT	WT	ND		ND	
C7	G13D	G13D	WT	WT	ND		ND	
C8	WT	WT	WT	WT	ND		ND	
C9	WT	WT	WT	WT	ND		ND	
C10	WT	WT	WT	WT	ND		ND	

[a]Allelic discrimination (codons 12 and 13), [b]Allelic discrimination (codon 600), [c]Allelic discrimination (codons 719, 790, 858 and 861), fragment analysis and Sanger sequencing (exons 19, 20 and 21), [d]fragment analysis and Sanger sequencing (exon 20). ND, not determined. Characters in bold show the discordance between routine and NGS analyses.

diagnosis at the same mutational hotspots. All the mutations detected in the routine diagnosis were also detected by NGS (Fig. 1 and Table II). Moreover, mutations not found in routine diagnosis were detected by NGS. These included a 15-nt deletion (c.2235_2249delGGAATTAAGAGAAGC) in two lung carcinomas classified as wild-type using routine methods

(patients L1 and L6). In the L1 sample, another mutation in the KRAS gene (G12D) was also identified. In patient L6, this 15-nt deletion in EGFR was concomitant with a V600E *BRAF* mutation.

In colon cancer, a 'common' 15-nt deletion in the EGFR gene was detected only with NGS. Up to now, rare mutations of the *KRAS* gene have not been routinely analyzed in lung carcinomas. In our small population, a Q61H mutation in the *KRAS* gene was found in the sample L8. This mutation was localized in exon 3, which is not routinely analyzed in lung cancer. No other alteration was found in the routinely analyzed genes (*HRAS* and *NRAS*).

In colon cancer, only the genes *KRAS*, *BRAF* and very recently *NRAS* and *HRAS* are studied. Concerning rare mutations of *KRAS*, *NRAS* and *HRAS*, we detected a Q61K mutation in the *NRAS* gene in patient C5. As numerous genes were sequenced by the Cancer Panel kit, we analyzed the results obtained for the *PIK3CA*, *HER2* and *EGFR* genes, which are routinely analyzed in lung carcinomas. No mutations were detected in exon 20 of *HER2*, or in exons 18, 20 or 21 of *EGFR*. In exon 20 of *PIK3CA*, an H1047L mutation was detected in patient C9. Concerning exon 19 of *EGFR*, a 15-nt deletion (the same as that observed in lung carcinomas) was detected in three patients (C4, C6 and C7). As this region was not routinely analyzed for colon cancer, we decided to perform both fragment analysis and Sanger sequencing. Neither fragment analysis, nor Sanger sequencing was able to detect the 15-nt deletion in exon 19 of *EGFR* in colon cancer (Fig. 2A). In contrast, NGS sequencing detected the deletion in >8% of sequenced fragments (Fig. 2B) for one patient. The two other patients harbored the mutation in approximately 4% of read sequences. Among these three patients, only one did not present a concomitant *KRAS* mutation.

WGA does not alter the NGS sequencing results. An important limitation in routine diagnosis is the quantity of gDNA extracted from FFPE samples and another paraffin block cannot be obtained in most cases. To counteract this limitation, we tested the impact of Whole Genome Amplification (WGA) on two samples of gDNA obtained from FFPE tissues. We then performed allelic discrimination on non-amplified gDNA and amplified gDNA (Fig. 3A). A *KRAS* G12C mutation was detected in both the amplified and non-amplified sample from patient L7. For patient L8, no *KRAS* G12C mutation was observed in either sample. In NGS analysis, the *KRAS* G12C mutation was also observed in patient L7 (non-amplified and WGA) but not in patient L8 (Fig. 3B). We also analyzed other routinely studied genes to compare the sequences before and after WGA. Whatever the gene analyzed, no point mutation was induced by the WGA (e.g., with the V600E *BRAF* and L858R *EGFR* hotpoint mutations in Fig. 3C). Even the rare mutation Q61H of KRAS was detected in both non-amplified and WGA gDNA from patient L8 (Fig. 3D). Moreover, the variant allele fraction was not modified after amplification.

NGS analysis revealed cancer susceptibility SNP and genetic alterations in some genes. The Illumina Cancer Panel kit studies exons with mutation hotpoints of 48 genes. We therefore analyzed all covered sequences for the 8 lung carcinomas and 10 colon carcinomas. Twenty-eight genetic alterations and three SNP related to cancer susceptibility or different protein activities were found (Table III). The most frequently altered gene was *TP53* with nine alterations detected in nine patients. Double mutations in the colon cancer susceptibility genes *APC* and *SMAD4* were detected in two patients (C3 and C8, respectively), suggesting a familial risk of colon cancer in these patients. For the patient with the *APC* mutation, we detected a concomitant *c-MET* activating mutation E168D. Concerning patient C8, we found a large number of alterations in different genes (*c-KIT*, *c-MET*, *FBXW7*, *FGFR3*, *FLT3*, *IDH1*, *KRAS*, *RB1*, *SMAD4* and *TP53*), suggesting high genetic instability in this *SMAD4* mutated tumor. Moreover, thanks to the non-targeted analysis, we detected two *BRAF* exon 15 mutations, N581S and V600R, which induce intermediate and strong activation of the protein, respectively. Furthermore, two mutations with unknown impact were detected in *PIK3CA* and *PTEN*. Concerning SNP, two patients (L7 and C4) harbored the breast cancer susceptibility *ATM* F858L SNP, and one patient (C10) had the rare *c-KIT* M541L SNP, which may influence the response to imatinib. Finally, 10 patients (8 heterozygotes and 2 homozygotes H472H) harbored the *KDR* Q472H polymorphism, which has been reported to increase tumor microvasculature.

Discussion

Molecular diagnosis is the current challenge in cancer management. Indeed, with the increased number of targeted therapies and resistance mechanisms developed by cancer cells, the molecular analysis of tumors is a very important task to achieve optimal cancer therapy. Sanger sequencing, even when accompanied by alternon-amplified technologies, such as allelic discrimination or high resolution melting technology, has shown its limits. Today, next-generation sequencing is providing exciting new perspectives. In this study, we tested Truseq Amplicon technology for the analysis of mutation hotspots of 48 genes in gDNA extracted from FFPE samples. All of the mutations detected by routine Sanger sequencing, allelic discrimination or fragment analysis (in *KRAS*, *BRAF*, *EGFR* genes) were also identified with NGS analysis. Moreover, other alterations at the mutation hotspots of the routinely analyzed genes were also detected. These additional alterations included a G12D *KRAS*, a V600R *BRAF* and a 15-nt deletion in exon 19 of *EGFR*. The additional deletion in the *EGFR* gene was concomitant with the G12D *KRAS* mutation in one patient, and with a V600E *BRAF* mutation in another patient. *KRAS, BRAF* and *EGFR* mutations are normally exclusive (9) but concomitant *KRAS* and *EGFR* mutations have already been described (10,11). The identification of concomitant mutations should increase with the higher sensitivity of NGS technologies. Nevertheless, as it may be impossible to confirm these 'new' mutations using routine techniques, their clinical relevance and even their existence may be debatable (12). In the same way, some mutations are detected by NGS in very few of the read sequences. For example, the 15-nt deletion found in three colon carcinomas was detected in less than 8% of the read sequences, and among these three colon carcinomas, one had no *KRAS*/*BRAF* mutation, one had a concomitant G12D *KRAS* mutation, and one also had

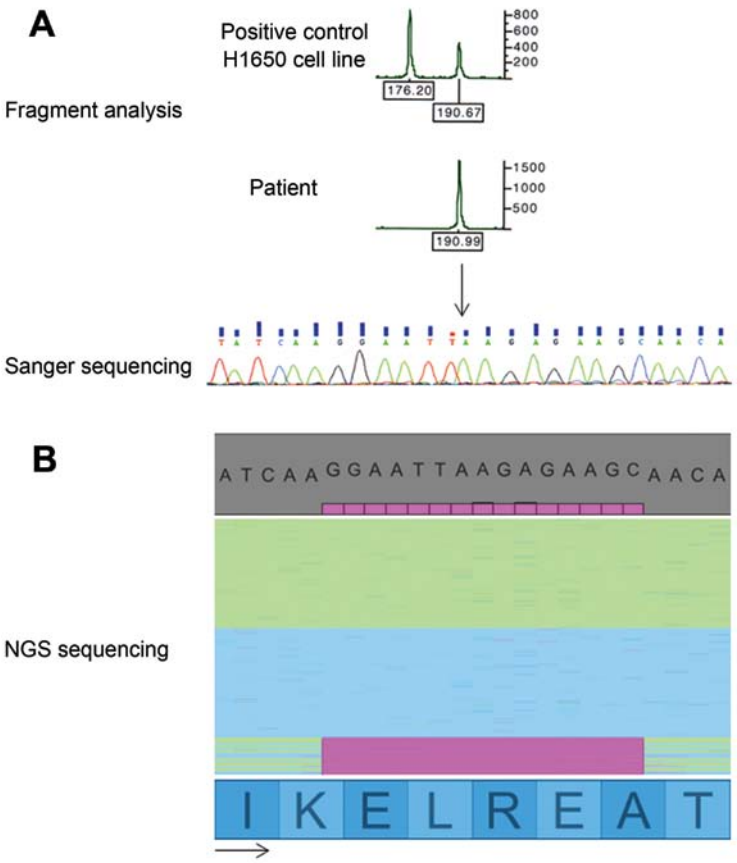

Figure 2. A 15-nt deletion detected in exon 19 of *EGFR* from colon carcinomas. (A) Fragment analysis and Sanger sequencing of *EGFR* exon 19 in a colon carcinoma. No alteration was observed in the region. (B) In the same sample analyzed with NGS, a 15-nt deletion (c.2235_2249delGGAATTAAGAGAAGC) was observed in about 8% of the read sequences. The arrow indicates the orientation of the gene.

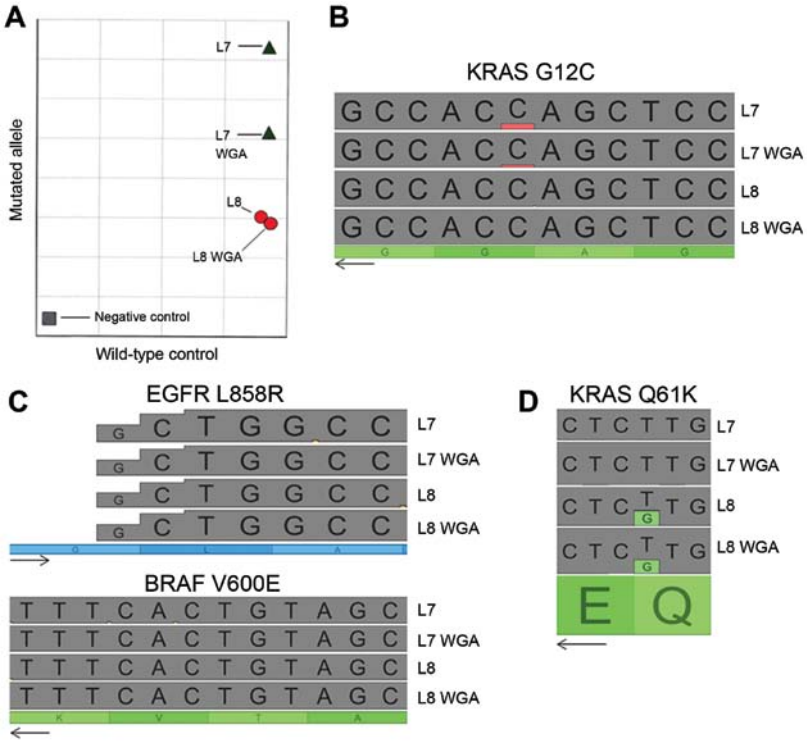

Figure 3. Whole Genome Amplification (WGA) of gDNA from FFPE did not alter the mutation profile of patients. (A) Routine G12C *KRAS* allelic discrimination of gDNA from patients L7 and L8 before and after WGA. (B) Analysis, by NGS, of *KRAS* exon 2 for the same patients. The profiles obtained with NGS technology are the same as with routine allelic discrimination: L7 WGA signal was lower than the non-amplified L7 signal. Nevertheless, this minor difference would not have affected the diagnosis. (C) WGA did not create genetic alterations, for example with the V600E *BRAF* and L858R *EGFR* hotpoint mutations. (D) The rare Q61K KRAS mutation observed in patient L8 was conserved by WGA. The arrows indicate the orientation of each gene.

Table III. Exonic SNP and genetic alterations in other analyzed genes.

Genes	Nucleotide variation	Protein sequence variation	Patients	Impact
APC	c.2626C→T	R876X	C3	Loss of function (familial mutation)
	c.3944C→T	S1315X	C3	Loss of function (somatic mutation) (26)
ATM	c.2572T→C	F858L	L7, C4	Breast cancer susceptibility SNP (23)
BRAF	c.1742A→G	N581S	C2	Intermediate activated (27)
	c.1798_1799GT→AG	V600R	C1	Strongly activated (28)
c-KIT	c.1621A→C	M541L	C10	SNP with a potential effect on imatinib response (29)
	c.2146G→A	D716N	C8	Possible resistance to imatinib (24)
c-MET	c.504G→T	E168D	C3	Activated (19)
	c.1156C→A	L386I	C8	Unknown (never observed)
FBXW7	c.832C→T	R278X	C8	Uncertain significance (30)
FGFR3	c.1196_1197GC→AG	R399H	C8	Unknown (31)
FLT3	c.2039C→T	A680V	C8	Activated (32)
IDH1	c.290G→A	G97V	C8	Loss of wild-type function (33)
KDR	c.1416A→T	Q472H	H: L1-L3, L5, L7-L8, C5-C6 O: C7, C9	Increased activity SNP (25)
KRAS	c.408T→A	S136K	C8	Unknown
PIK3CA	c.2176G→A	E726K	C2	Unknown (34)
PTEN	c.563A→T	D187V	L1	Unknown
RB1	c.2074_2075insATGA	Y692FsX2	L2, L5	Loss of function
	c.2119T→C	S707P	C8	Unknown
SMAD4	c.1009G→A	E337K	C8	Unknown
	c.1082G→A	R361H	C8	Loss of function (35)
TP53	c.310C→T	Q104X	L1	Unknown[a]
	c.523C→T	R175V	C8	Unknown
	c.527G→T	C176F	L5	Partially functional/deleterious[a]
	c.536A→G	H179R	C3	Non-functional/deleterious[a]
	IVS5+2T→G	G187Fs	C1	Unknown
	c.709A→C	M237L	C10	Partially functional/deleterious[a]
	c.743G→A	R248Q	C6	Non-functional/deleterious[a]
	c.742C→T	R248W	C5	Non-functional/deleterious[a]
	c.830G→T	C277F	C7	Non-functional/deleterious[a]

[a]From IARC database (36).

a G13D *KRAS* mutation. This observation is quite disturbing, and raises two questions: was the 15-nt deletion true, and if so, was this alteration clinically relevant given the small number detected. Today, the only way to have an answer would be to treat these patients with EGFR tyrosine kinase inhibitors or to observe anti-EGFR antibody resistance in these patients. To date, only patients with *KRAS* (13), *HRAS* or *NRAS* mutations (7,8) can be diagnosed as immediately resistant. Concerning our three colon carcinomas, two may benefit from treatment with EGFR TKI as the G13D *KRAS* mutation does not seem to interfere with the inhibition of the EGFR pathway (14).

With the increase in the number of genes to be analyzed for molecular diagnosis, the quantity of gDNA obtained from FFPE tissues will rapidly become a major problem, especially for lung carcinomas. In this work, we tested Whole Genome Amplification in two lung carcinomas and analyzed the

resulting samples by NGS. The genetic profile obtained before and after WGA was qualitatively the same and quantitatively close. Indeed, only the intensity of the G12C *KRAS* mutation in patient L7 was slightly lower in the amplified sample. The strong similarity between amplified and non-amplified samples is in accordance in very recent studies, which showed that WGA can be safely used for diagnosis (15,16). Moreover, through this experiment, we showed that Truseq Amplicon technology is compatible with samples treated by WGA.

Among the genes or codons studied in the panel but not analyzed in routine molecular diagnosis, we detected 27 different alterations in 14 genes. Of these, 9 were detected in the *TP53* gene, which is the most frequently altered gene in cancer (17). Eight mutations were in the DNA binding domain of the protein, indicating that these mutations are deleterious. One mutation occurred in a splice site, inducing a frameshift that may not be deleterious (18). We detected 2 genes with a double mutation, *APC* and *SMAD4*. The presence of two mutations in these two colon cancer predisposition genes indicated that these patients could have been members of families with a high risk of colon cancer. Both patients harbored mutations in the c-MET genes. The APC mutated patient had the activating mutation E168D (19), making him/her eligible for crizotinib therapy, which is generally used in lung cancer (20). Concerning the SMAD4 mutated tumor, we detected eight other altered genes, suggesting high genetic instability in this tumor type (21,22). The impact of most of these alterations is unknown. Nevertheless, the activating A680V mutation of FLT3 may be targeted by anti-FLT3 therapies currently in clinical development for the treatment of leukemia.

Three SNP modifying protein sequences were found. *ATM* F858L polymorphism, detected in two patients, is associated with an increased risk of breast cancer (23), but the small number of patients in our study does not allow us to draw any conclusions with regard to the predisposition for colon and lung cancer. Then, *c-KIT* M541L polymorphism was found in only one patient. The impact of this polymorphism in not known, but it has been suggested that it may affect the response to imatinib (24). Finally, KDR Q472H polymorphism was the most interesting alteration. Indeed, tumors with histidine show higher vascularization than do tumors with glutamine (25). In the Caucasian population, the frequency of each genotype is 58, 36 and 6% for Q472Q, Q472H and H472H, respectively. In our small population of tumors (n=18), we found enrichment of the histidine allele in 55.5% of our tumors (44.5% Q/Q, 44.5% Q/H and 11% H/H). In lung carcinomas we observed an enrichment of heterozygous tumors (75%), whereas in colon, the enrichment concerned homozygous H/H tumors. Moreover, during the analysis, we found that the presence of each allele was not 50/50 in heterozygous tumors, but varied from 9 to 96% of the read sequences. This raises the question of true polymorphism or a selection of tumor cells with a high angiogenic capacity. To answer this question, it would be necessary to analyze the constitutive DNA of each patient. According to the study by Glubb *et al*, patients with heterozygous or homozygous histidine tumors could be more sensitive to inhibiting treatments of VEGFR2 or to bevacizumab.

In conclusion, the analysis by NGS of FFPE lung and colon carcinomas identified the alterations highlighted by routine molecular diagnosis techniques. Thanks to its higher sensitivity, NGS analysis revealed new mutations that were not detected routinely. The impossibility to confirm the presence of these mutations by another technology is problematic, and the only way to answer this question is by conducting clinical trials that compare treatments of patients diagnosed by routine techniques or by NGS. Finally, the use of NGS in routine practice could revolutionize the management of cancer patients. Indeed, simultaneous analysis of numerous genes could identify drug-sensitive alterations generally observed in other cancer types (for example a *c-MET* alteration in a colon carcinoma that would be treated with crizotinib in lung cancer). Nevertheless, high throughput studies that combine NGS analysis and clinical trials need to be performed before NGS analysis can be generalized in routine molecular diagnosis.

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