Detection of K-Ras mutations in tumour samples of patients with non-small cell lung cancer using PNA-mediated PCR clamping

Non-small cell lung cancers (NSCLC), in particular adenocarcinoma, are often mixed with normal cells. Therefore, low sensitivity of direct sequencing used for K-Ras mutation analysis could be inadequate in some cases. Our study focused on the possibility to increase the detection of K-Ras mutations in cases of low tumour cellularity. Besides direct sequencing, we used wild-type hybridisation probes and peptide-nucleic-acid (PNA)-mediated PCR clamping to detect mutations at codons 12 and 13, in 114 routine consecutive NSCLC frozen surgical tumours untreated by targeted drugs. The sensitivity of the analysis without or with PNA was 10 and 1% of tumour DNA, respectively. Direct sequencing revealed K-Ras mutations in 11 out of 114 tumours (10%). Using PNA-mediated PCR clamping, 10 additional cases of K-Ras mutations were detected (21 out of 114, 18%, P < 0.005), among which five in samples with low tumour cellularity. In adenocarcinoma, K-Ras mutation frequency increased from 7 out of 55 (13%) by direct sequencing to 15 out of 55 (27%) by clamped-PCR (P < 0.005). K-Ras mutations detected by these sensitive techniques lost its prognostic value. In conclusion, a rapid and sensitive PCR-clamping test avoiding macro or micro dissection could be proposed in routine analysis especially for NSCLC samples with low percentage of tumour cells such as bronchial biopsies or after neoadjuvant chemotherapy.

Keywords: non-small cell lung cancer; K-Ras mutations; sensitivity; real-time PCR; PNA

Malignant transformations are the result of an accumulation of carcinogenesis steps corresponding to activation of oncogenes and inactivation of tumour suppressor genes (Bishop, 1991). Among the available candidates, the K-Ras proto-oncogene is the most well-studied cellular gene whose alterations seem to have an important role in the pathogenesis of human cancer. K-Ras oncogene is a known downstream signaling molecule in the EGFR-signaling pathway. K-Ras gene encodes a 21 kDa GTP-binding protein, which controls the mechanisms of cell growth and differentiation. Point mutations in the K-Ras gene lead to uncontrolled stimulation of Ras-related functions by altered p21ras protein, locking it in the ‘on’ position for signal transduction (Adjei, 2001; Molina and Adjei, 2007).

Non-small cell lung cancers (NSCLC) represent more than 80% of lung cancers and are subgrouped in squamous cell carcinomas (SCC), adenocarcinoma (ADC) and large cell carcinoma (Travis et al, 2004). K-Ras mutations are found in 10–20% of NSCLC and have been described in approximately 30% of ADC (Ahrendt et al, 2001). About 92% of K-Ras mutations occur in codon 12 (Hunchak et al, 1999). K-Ras mutations are most closely associated with a history of cigarette smoking and are more common in women (Ahrendt et al, 2001; Broermann et al, 2002; Sugio et al, 2006; Tam et al, 2006). Oncogenic activation of K-Ras has been reported as a prognostic marker of poor outcome in NSCLC patients and K-Ras mutations seemed to be associated with a shorter survival in early-stage and locally advanced NSCLC (Pukuyama et al, 1997; Hunchak et al, 1999). A recent meta-analysis of K-Ras mutations in lung cancer showed that these mutations appeared to be associated with shorter survival in NSCLC (Mascaux et al, 2005). It was also suggested that K-Ras mutations could be predictive of chemotherapy resistance, in metastatic disease rather in adjuvant situation (Eberhard et al, 2005; Tsao et al, 2007). Although meta-analyses has indicated that K-Ras gene mutations are weak prognostic markers of poorer outcome in NSCLC, results from individual studies have been inconsistent (Hunchak et al, 1999; Mascaux et al, 2005; Tsao et al, 2007).

At first, EGFR status is a decisive molecular factor for using EGFR-targeted therapies in NSCLC (Lynch et al, 2004; Paez et al, 2004; Pao et al, 2004). K-Ras and EGFR mutations were shown to
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Table 1 Patients' characteristics

|                | All NSCLC | ADC/BAC | SCC |
|----------------|-----------|---------|-----|
| Age (years): mean (s.d.) | 61 (10)   | 61 (10) | 61.5 (10) |
| Gender          |           |         |     |
| Men             | 91 (80%)  | 37 (68%)| 54 (91.5%)|
| Women           | 23 (20%)  | 18 (33%)| 5 (8.5%)  |
| Current/former smoker | 106 (93%) | 47 (87%)| 59 (100%) |
| Never-smoker    | 7 (6%)    | 7 (13%) | 0 (0%)   |
| Stage pTNM      |           |         |     |
| I—IIA           | 99 (87%)  | 45 (82%)| 54 (91.5%)|
| III—IVa         | 15 (13%)  | 10 (18%)| 5 (8.5%)  |
| Histologic differentiation |       |         |     |
| Well/moderate   | 74 (65%)  | 28 (57%)| 46 (80%)  |
| Poor            | 32 (28%)  | 21 (43%)| 11 (19%)  |
| Tumour cell count |         |         |     |
| <50%            | 52 (46%)  | 28 (51%)| 24 (41%)  |
| ≥50%            | 62 (54%)  | 27 (49%)| 35 (59%)  |
| Lung cancer relapse |       |         |     |
| Yes             | 52 (45%)  | 28 (54%)| 23 (39%)  |
| No              | 60 (53%)  | 24 (46%)| 36 (61%)  |
| Lung cancer death |         |         |     |
| Yes             | 53 (46.5%)| 28 (53%)| 25 (42%)  |
| No              | 59 (52%)  | 25 (47%)| 34 (58%)  |

NSCLC = non-small cell lung cancer; ADC = adenocarcinoma; BAC = bronchiolo-alveolar carcinoma; SCC = squamous cell carcinoma. Clinical and pathological features of 114 NSCLC patients with NSCLC, classified by histological subtypes.
*Of whom eight BAC features. **Stages IV with unique metastasis.

MATERIALS AND METHODS

Patients

Anonymised frozen samples from 114 routine consecutive NSCLC patients surgically treated were obtained from the Biological Resource Center of the University Hospital of Strasbourg, in protocols approved by the institutional review board. Stage was defined as recommended (Moutain, 1997). All patients are chemotherapeutic drug-naive at the time of surgery. All the patients are non-Asian. The patients included 91 men and 23 women. Only seven (6%) of the patients were never smokers. The observation period ranged from 1 to 82 months, with a median follow-up of 26 months. Patients’ characteristics are summarized in Table 1. Tumour and paired normal lung peripheral tissue samples obtained at the time of surgery, were immediately stored at −80°C. Tumours were histologically classified according to the World Health Organization guidelines and scored for differentiation (Travis et al, 2004). Three haematoxylin and eosin-stained sections of frozen tissues were reviewed by a pathologist to evaluate the percentage of tumour cells, at the beginning, middle and end of the frozen tissues. The percentage of tumour cells corresponds to the number of tumour cells reported to the number of all the cells (tumour and non-tumour cells) analysed on a slide. Almost half of the patients presented less than 50% of tumour cells. Histological characteristics are summarized in Table 1.

DNA extraction from frozen lung cancer tumours

Genomic DNA was isolated using conventional techniques with QIAamp DNA kit (Qiagen, Courtabeuf, France). To validate the percentage of tumour cells assessed by the pathologist in the
Frozen samples, paired tumour and normal DNA were amplified by fluorescent polymerase chain reaction (PCR) for microsatellite (MS) analysis with a panel of markers distributed among the genome on 13 chromosomes frequently altered in NSCLC (data not shown). Detailed MS and PCR conditions are available upon request.

**DNA sequencing analysis**

The mutational status of K-Ras (codon 12,13) was performed using 40 ng of genomic DNA amplified by PCR in 25 μl reaction mix containing 1.25 U Fast Start High Fidelity Taq (Roche), 0.2 mM dNTP, 1.5 mM MgCl2, and 0.2 μM of K-Ras F and K-Ras R I primers (Table 2). After an initial denaturation (2 min at 94°C), a touch down protocol was used as follows: 10 cycles with a decreased hybridisation temperature from 61 to 58°C. The subsequent 35 cycles were performed as follows: 50 s at 93°C, 50 s at 55°C, and 1 min 30 s at 72°C, followed by a final extension period of 10 min at 72°C. All PCR products were verified by electrophoresis on agarose gels. After purification (Microcon-PCR Filter Unit, Millipore, Paris, France), the PCR products were sequenced (BigDye Terminator v1.1 Cycle sequencing kit, Applied Biosystems, Forster City, CA, USA) and analysed on ABI PRISM 3100 Genetic Analyser (Applied Biosystems). The GB sequence of human K-Ras (L00045) was used as a reference for sequence analysis (Seqscape v2.5, Applied Biosystems). All sequencing reactions were performed in both forward and reverse directions, and all mutations were confirmed by sequencing a second independent PCR product. As expected, by direct sequencing technique for K-Ras mutation analysis, K-Ras mutations were reproducibly detectable at a dilution of 25% of mutated DNA into normal DNA (data not shown). All the mutated K-Ras DNA were also sequenced for EGFR mutation analysis as described earlier (Beau-Faller et al, 2008).

**Real-time PCR and melting curve analysis, clamped-probe assay**

Primers were chosen to amplify a specific 243-bp genomic fragment from K-Ras codon 12 and 13. Hybridisation FRET probes were designed complementary to wild-type sequence of codon 12 and 13. The anchor probe was 5′-labeled with LC-Red 640 and 3′ phosphorylated, and the sensor probe was 3′-labeled with fluorescein. The PNA oligomer covered codons 10–14 wild-type sequence. Sequences of primers (Eurogentec, Liège, Belgium), probes (Timolbiol, Berlin, Germany), and 17 mer-PNA (Eurogentec, Liège, Belgium) are listed in Table 2. They are represented in Figure 1. Real-time PCR was performed with 20 ng of genomic DNA, in a final volume of 20 μl containing Light Cycler Fast Start DNA Master Hyb Probe (Roche Diagnostics, Mannheim, Germany), 3 mM MgCl2, 0.4 μM of each primers K-Ras, 0.4 μM of K-Ras hybridisation sensor probe and 0.2 μM of K-Ras

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**Table 2** DNA sequences of primers, PNA oligomer, and probes for detecting K-Ras mutations

| Name     | DNA (5′–3′) or PNA (NH2-CONH2) sequence* | Position b nt |
|----------|-----------------------------------------|--------------|
| K-Ras F  | GGAGATTTTGATGGATATTACCT                  | 9–33         |
| K-Ras R I| AGAATTGAGTTGAGTATGATTATG                | 251–236      |
| K-Ras R II| GTCCGTACAGAGTAATACTGC                  | 244–226      |
| Anchor   | LC-Red 640-ACTACACA                     | 121–86       |
| Sensor   | AGTTTAATACGGACTATTTTCAGGG-Ph            |              |
| PNA      | CCTACGCCACCATGCT-Flu                    | 138–124      |
| PNA      | CCTACGCCACCATGCTCC                      | 138–122      |

*The 3′ end of the anchor probe was phosphorylated to prevent probe elongation by Taq polymerase during PCR. Underlined, nucleotide complementary to wild-type bases of codon 12 and 13. The base numbering is according to GenBank accession no L00045. aF = forward; R = reverse; Flu = fluorescein; LC-Red = LightCycler-Red; Ph = phosphorylated; bThe numbering is according to GenBank accession

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**Figure 1** Schematic representation using PNA-mediated PCR for detection of mutant K-Ras. Relative positions of PCR primers (K-Ras F, K-Ras R), wild-type hybridisation probes (Anchor, Sensor) and peptide-nucleic-acid (PNA) for K-Ras mutations detection at codons 12 and 13.
hybridisation anchor probe, without or with 0.1 μM PNA oligomer. After the initial denaturation step at 95°C for 10 min, a touch down amplification was performed consisting of a denaturation at 95°C for 10, 7 s at 76°C, then 10 cycles with touchdown annealing for 15 s from 65 to 55°C (decreasing 2°C/two cycles) and elongation at 65°C for 20 s. This step was followed by 28 cycles: 95°C for 10 s, 76°C for 7 s, 50°C for 15 s, 65°C for 20 s. Melting curve analysis was performed by increasing temperature from 40 to 95°C with a transition rate of 0.25°C/s. Fluorescence data were analysed using the Light Cycler software (software version 3.5, Roche Diagnostics). Mutation analysis for each tumour sample was performed at least two times. PNA-PCR products from all samples that gave positive results by the clamped-probe assay were sequenced to confirm and precise the type of K-Ras mutation.

### Controls

DNA from a lung cancer cell line A549 with a homozygous K-Ras codon 12 mutation (c.34G > A, G12S) was used for homozygous K-Ras mutation positive control. One negative control (DNA from colon cancer cell line HT29 of colon carcinoma with wild-type K-Ras sequence) and a no template negative control (as control for contamination) were processed in parallel. We evaluate the sensitivity of the developed alternative methods by serially diluting DNA from A549 mutated K-Ras cell line in the DNA of wild-type K-Ras HT29 cell line (Figure 2).

### Statistical analysis

A total number of patients to be tested by PNA-PCR clamp was decided to be more than 100 patients because about 15–20% of NSCLC patients were reported to have K-Ras mutations in previous articles (Mascaux et al., 2005). Statistical analysis using chi² test or exact test (type Fisher’s test) if appropriate with exact P-values was used to compare the qualitative data. Where appropriate, continuous variables were categorized before analysis. The date of point was 30 April 2008. The Kaplan–Meier method was used to estimate the probability of event-free survival and the log-rank test to detect the difference in survival curves. Cox’s proportional hazard models were used to determine the impact of patient characteristics on event-free survival. All statistical calculations were performed with the Statistical Package for the Social Science (SPSS) (number 15.0) statistical software.

### RESULTS

#### Sequence analysis of NSCLC samples

We first analysed the 114 routine samples by direct sequencing for K-Ras mutation detection. By this technique, 11 (10%) of the 114 cases showed a K-Ras mutation at codon 12 or 13, represented by five mutations G12C, two mutations G12D, two mutations G12V, one mutation G12R and one mutation G12A (Tables 3 and 4).

#### Determination of the sensitivity of the hybridisation-probe assay and clamped-probe assay

The tumour cell count was lower than 50% in 52 patients, and among them lower than 25% in 15 patients (Table 1). To further improve the detection of K-Ras mutations in comparison with direct sequencing, we developed real-time PCR with wild-type hybridisation probes without or with PNA-mediated clamped PCR.

### Table 3

| (Codon)mutation* (amino acid change) | Sequencing (%)b | Hybrisation (%)b | PNA (%)b |
|--------------------------------------|-----------------|-----------------|-----------|
| (12) GGT → AGT (Gly → Ser)           | 0 (0)           | 0 (0)           | 1 (5)     |
| (12) GGT → GCT (Gly → Arg)           | 1 (9)           | 1 (6)           | 1 (5)     |
| (12) GGT → GTG (Gly → Cys)           | 5 (45.5)        | 8 (50)          | 10 (46)   |
| (12) GGT → GAT (Gly → Asp)           | 2 (18)          | 3 (19)          | 5 (24)    |
| (12) GGT → GCT (Gly → Ala)           | 1 (9)           | 1 (6)           | 1 (5)     |
| (12) GGT → GTT (Gly → Val)           | 2 (18)          | 2 (12.5)        | 2 (9.5)   |
| (13) GCC → GGC (Gly → Cys)           | 0 (0)           | 1 (6)           | 1 (5)     |

*Altered bases are underlined. b(%) Percentage of mutation type reported to mutated cases.

### Table 4

| No | Sex | Age (years) | Histological type | Tumour cells count (%) | Stage | Relapse | EFS (months) | Technique using for K-Ras mutation detection | Type of K-Ras mutation |
|----|-----|-------------|-------------------|------------------------|-------|---------|-------------|---------------------------------------------|------------------------|
| 77 | M   | 51          | ADC               | 90                     | IIIB  | Yes     | 2           | SQC                                        | G12C                   |
| 108| F   | 52          | ADC               | 70                     | IIIA  | Yes     | 2           | SQC                                        | G12C                   |
| 118| M   | 68          | SCC               | 60                     | IB    | No      | 25          | SQC                                        | G12D                   |
| 24 | M   | 58          | ADC               | 60                     | IB    | No      | 39          | SQC                                        | G12C                   |
| 1109| M     | 60         | ADC-BAC           | 50                     | IIIB  | No      | 52          | SQC                                        | G12A                   |
| 50 | M   | 54          | SCC               | 50                     | IA    | Yes     | 8           | SQC                                        | G12C                   |
| 1124| F     | 44         | ADC               | 50                     | IIIA  | Yes     | 14          | SQC                                        | G12D                   |
| 1104| M     | 70         | ADC-BAC           | 40                     | IIIIB | No      | 44          | SQC                                        | G12C                   |
| 1118| F     | 53         | ADC               | 40                     | IV    | Yes     | 1           | SQC                                        | G12C                   |
| 9915| M    | 60          | SCC               | 40                     | IIIB  | Yes     | 3           | SQC                                        | G12D                   |
| 22013| F    | 60         | SCC               | 90                     | IB    | No      | 18          | HYB                                        | G12C                   |
| 52 | M   | 44          | ADC               | 70                     | IIIA  | Yes     | 22          | HYB                                        | G12C                   |
| 63 | F   | 44          | ADC               | 50                     | IA    | No      | 49          | HYB                                        | G12D                   |
| 91 | M   | 67          | ADC               | 15                     | IB    | No      | 18          | HYB                                        | G12C                   |
| 1126| M    | 74         | ADC               | 1                      | IA    | No      | 29          | HYB                                        | G13C                   |
| 1116| M    | 55         | ADC               | 50                     | IIIB  | No      | 26          | PNA                                        | G12S                   |
| 22006| M   | 56         | ADC               | 50                     | IIIA  | Yes     | 14          | PNA                                        | G12C                   |
| 1103| M    | 76         | SCC               | 10                     | IB    | No      | 8           | PNA                                        | G12D                   |
| 121 | M   | 45          | SCC               | 1                      | IB    | No      | 49          | PNA                                        | G12C                   |
| 102 | M   | 56          | ADC               | 1                      | IIIA  | Yes     | 39          | PNA                                        | G12D                   |

ADC = adenocarcinoma; BAC = bronchioloalveolar carcinoma; SCC = squamous cell carcinoma; EFS = event-free survival; SQC = results obtained by direct sequencing; HYB = results obtained by hybridisation probe assay; PNA = results obtained by clamped-probe assay.
We assessed the sensitivity of these assays by testing homozygous G12S K-Ras mutated A549 cell line DNA serially diluted into DNA from wild-type K-Ras HT29 cell line DNA. By hybridisation-probe assay, the mutations were reproducibly detectable at a dilution of 10% of mutated DNA into wild-type DNA and by the clamped-probe assay, at a dilution of 1% of mutated DNA into wild-type DNA (Figure 2). Indeed, in our conditions, addition of PNA allows to increase the threshold detection of K-Ras mutation by a magnitude of 10.

Hybridisation-probe and clamped-probe assays for detecting K-Ras mutations. Comparison with direct sequencing

All the 114 patients were then analyzed for K-Ras mutations by hybridisation-probe assay and clamped-probe assay (Table 2). As expected, the 11 out of 114 (10%) tumours with a K-Ras mutation at codon 12 or 13 detected by direct sequencing, were also detected by hybridisation-probe/clamped-probe assays. However, five tumours with wild-type K-Ras assessed by direct sequencing presented K-Ras mutations when using the hybridisation-probe assay. Remarkably, five other tumours with wild-type K-Ras assessed by direct sequencing and by the hybridisation-probe assay presented K-Ras mutations when using clamped-probe assay alone. As expected, all the positive samples detected by hybridisation-probe assay were also positive for clamped-probe assay. Thus, these sensitive techniques allowed an increase in detection of K-Ras mutation from 11 out of 114 patients (10%) to 21 out of 114 (18%) (P<0.005). To confirm the mutations in the 10 tumours only detected by these sensitive techniques and to identify precisely the type of K-Ras mutation, all PNA – PCR products were directly sequenced for codon 12 and 13 of K-Ras. We identified five mutations G12C, three mutations G12D, one mutation G12S and one mutation G13C with hybridisation-probe/clamped-probe assays (Tables 3 and 4, Figure 3).

Among the K-Ras mutated tumours diagnosed only by hybridisation/PNA techniques, five had a percentage of tumour cells effectively lower than required for classical sequencing. The tumour cellularity of these tumours was 15% (one patient), 10%

![Figure 3](image-url) Representative melting curves obtained by hybridisation and clamped-probe assays. On the right side, paired electropherograms obtained by sequencing of a PCR product, without and with PNA. (A) K-Ras mutation (G12D) diagnosed by the two alternative techniques: hybridisation probe and clamped-PCR assays (No. 63). (B) K-Ras mutation (G12D) only diagnosed by the most sensitive technique: clamped-PCR (No. 1103).
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DISCUSSION
Surgical tumour specimens of NSCLC may contain a lot of normal/inflammatory cells. The presence of a high percentage of normal/inflammatory cells could lead to ‘false-negative’ results when mutations were detected by direct sequencing. A recent commentary synthetically addresses the question of the ideal method for EGFR mutation testing in lung cancer (Pao and Ladanyi, 2007), but no such data are available for K-Ras. We developed a quick, cheap and sensitive method for detecting K-Ras mutations from routine surgical specimens of NSCLC without removing contaminating normal cells by macro/micro dissection.

PNA–PCR clamp method can rapidly (within 2 h) detect K-Ras mutations using a low quantity of DNA. By contrast to other PNA studies, we used wild-type fluorescent-labelled hybridisation probes allowing rapid and high-sensitive detection of all the K-Ras mutations (Sun et al, 2002; Chen et al, 2004; Taback et al, 2004; Däbritz et al, 2005; Luo et al, 2006; Miyake et al, 2007). Only one pair of primers and one pair of probes are required to detect all possible mutations in codons 12 and 13 of the K-Ras gene. The detected fluorescence signal corresponds to the amplified mutant DNA and can be analysed by a subsequent melting curve analysis. If requested, the PNA–PCR products are available to precisely identify the mutated nucleotide. All these advantages greatly simplify the manipulating procedure.

The sensitivity of direct sequencing depends on the percentage of tumour cells in the analysed sample, generally up to 50% tumour cells is requested with a detection threshold of 25% mutant DNA for hybridisation-probe assay and for clamped-probe assay, respectively, and might correspond to a more sensitive test, applicable for routine purposes without macro or micro dissection. Another new rapid method for mutated EGFR and K-Ras detection by high resolution melting analysis (HRM) has been recently reported (Do et al, 2008). Even HRM is a promising method, it can be compromised by a low proportion of tumour cells in the analysed sample and by the difficulty to detect homozygous mutations, as the sensitivity of K-Ras mutations detection was only 5–10%. Among the 10 new K-Ras mutated patients detected by these most sensitive methods, half presented a percentage of tumours under the level usually required for direct sequencing. Thus, even in a surgical cohort of NSCLC tumour samples, there is a risk of false-negative K-Ras mutated patients when they were analysed by direct sequencing. The rapidity and sensitivity of our sensitive technique could lead us to propose this test for routine K-Ras mutation detection, that is, bronchial biopsies in NSCLC.

In our study, the 10 new K-Ras-mutated cases presented clinical/biological characteristics usually associated with K-Ras mutations.
in NSCLC (Ahrendt et al, 2001; Broermann et al, 2002; Sugio et al, 2006; Tam et al, 2006). Remarkably, none of the five new K-Ras mutated patients with more than 50% of tumour cells, presented an EGFR mutation (exon 18, 19, 20, 21) (data not shown). Interestingly, if K-Ras mutations diagnosed by direct sequencing appeared to be a prognostic marker of poorer outcome in our cohort of surgically treated NSCLC patients, this tendency is not more observed when we considered K-Ras mutations diagnosed by the two other techniques (hybridisation probe alone, or with clamped-PCR). Our survival analysis is nevertheless limited by the relatively short follow-up with fewer than half of the patients having relapsed or died. Accordingly, a recent study (JBR.10) showed that mutations of K-Ras gene were neither prognostic for survival nor predictive of a differential benefit from adjuvant chemotherapy in stage IB and II NSCLC (Tsao et al, 2007). In this study, the tumour cellularity was enriched by microdissection, and using allelic-specific oligonucleotide hybridisation, they failed to confirm K-Ras oncogenic activation as a significant marker of poor prognosis after surgery for NSCLC. The lack of prognostic value of all the K-Ras mutated cases could be explained by the fact that in cases of mutation detected by sensitive techniques, the real percentage of K-Ras mutated cells could be too low, under a threshold with immediate clinical significance. Further studies are required to precisely determine the significant prognostic threshold of K-Ras mutated cells.

In our study, half of the new K-Ras mutated cases diagnosed only by sensitive techniques, presented a percentage of tumour cells above the threshold usually required for direct sequencing. These results suggest the presence of mutated subclones in the tumour. In fact, direct sequencing could fail to detect mutated subclones (Pao and Ladanyi, 2007). Some EGFR-mutated subclones were already detected in NSCLC when EGFR T790M mutations have been analysed with sensitive techniques (Inukai et al, 2006). It was suggested that the possible presence of such mutations at a low frequency in NSCLC tumours before EGFR-targeted therapy might affect the tumour response or the event-free survival after targeted treatments. Recently, K-Ras mutations detected by allelic discrimination on tumour DNA, have been demonstrated to be an independent prognostic factor in patients with advanced colorectal cancer treated with cetuximab (Lièvre et al, 2008). To our knowledge, no predictive studies have been realised to identify K-Ras mutated subclones in NSCLC, but the presence of such mutated subclones could explain some cases of secondary EGFR-targeted therapy resistance. Therefore, it would be important to evaluate the predictive value of such K-Ras mutated tumour subclones, particularly in stabilised or progressive cases treated by targeted EGFR therapy.

Consequently, the percentage of tumour cells in the analyzed sample as well as the threshold of mutation detection of the currently molecular test appeared to be two essential data to better understand the significance (i.e., prognostic or predictive factor) of the detected mutation. Indeed, in our cohort of surgical specimen, 53 (46.5%) of our routine consecutive patients presented a percentage of tumour cells under the value (50%) classically required for direct sequencing; and in addition, some patients with a percentage of tumour cells sufficient for direct sequencing, appeared to have K-Ras mutations at a low frequency. In the first type of tumours, a sensitive technique will detect low level of K-Ras mutations with finally a number of K-Ras mutated tumour cells which could have clinical usefulness; in the second type of tumours, sensitive technique will detect K-Ras-mutated subclones of which clinical usefulness has still to be explored.

In conclusion, our study allows us to propose an easy and sensitive method for rapid analysis of K-Ras mutations in NSCLC tumours. This sensitive technique could be helpful for speciments of lung cancer without removing contaminating normal cells, mostly in the setting of NSCLC, in which diagnoses are often based on bronchial biopsies or cytologic specimens. The prognostic value of such sensitive detected K-Ras mutations when tacking into account the tumour cellularity, has still to be evaluated on an increased number of surgically treated lung cancer patients, taking into account the frequency of mutated allele in the sample depending on the tumour cellularity. Furthermore, predictive value of low represented K-Ras mutations need to be assessed in lung cancer patients treated by targeted anti-EGFR treatments, to optimise such treatments in NSCLC patients.

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