Validation of the Point-EXACCT method in non-small cell lung carcinomas

Veerle A.M.C. Somers,1* Darcy A. Leimbach,2 Paul H.M.H. Theunissen,3 James J. Murtagh, Jr.,2 Brian Holloway,4 Anton W. Ambergen,5 and Frederik B.J.M. Thunnissen1

K-ras point mutations are often detected in part of the lung carcinomas. For the validation of a highly sensitive and rapid assay for known point mutations, Point-EXACCT (Biochim Biophys Acta 1998; 1379:42–52), we analyzed 89 non-small cell lung carcinomas and compared the results with two sequencing methods. No point mutations were found with double-stranded sequencing. Single-stranded sequencing detected six patients positive for K-ras codon 12. When Point-EXACCT was used, K-ras codon 12 mutations were detected in 8 of 52 patients with squamous cell carcinomas, 10 of 29 patients with adenocarcinomas, and 3 of 8 patients with large cell carcinomas. The finding of K-ras mutations in squamous cell carcinomas is explained by the high sensitivity of the method. Therefore, Point-EXACCT may be applicable to detection of those alterations occurring at a low frequency among an excess of cells with wild-type DNA.

Point mutations in the Kirsten ras (K-ras) oncogene are one of the most common genetic alterations involved in various types of human cancer (1, 2). In lung cancer, K-ras mutations occur predominantly in codon 12 (3–7). The frequency of those alterations varies within different histological subtypes. K-ras point mutations are found in approximately 15–56% of the adenocarcinomas and to a lesser extent in other types of non-small cell lung carcinomas (NSCLCs) (3, 5, 7–14). The timing of K-ras activation in the process of lung cancer development is still unclear. Both human and animal studies have initially reported that K-ras mutations arise early (15–17), i.e., just before the occurrence of visible tumors. Compared with far more early chromosomal deletions demonstrated in heavy smokers, the K-ras mutation seems to be a late event (18, 19).

Several methods have been utilized for the detection of K-ras point mutations in lung cancer. First reports on K-ras point mutation analysis used relatively insensitive methods such as allele-specific oligonucleotide hybridization or direct sequencing of amplification products (3, 4, 8, 10, 20–25). At the moment, a number of methods displaying a considerable increase in sensitivity of point mutation detection have been described. Those methods include amplification refractory mutation system (26), allele-specific amplification (27), mismatch amplification mutation assay (28), oligonucleotide ligation assay (29), ligation chain reaction (30), restriction fragment length polymorphism (31), enriched PCR (32), PCR-primer-introduced-restriction analysis with enrichment of mutant alleles (33), PCR-based cloning and hybridization (34), and point mutation detection using exonuclease amplification coupled capture technique (Point-EXACCT) (35, 36). Interestingly, in those studies, the percentage of cases positive for K-ras seemed to be dependent on the sensitivity of the different methods used for point mutation detection.

In general in tumor tissue, a variable number of nonmalignant cells, such as stromal and inflammatory cells, are mixed with tumor cells. In some tissue samples, the fraction of tumor cells may occasionally be <10%. However, this fraction is usually >40%. Many of the methods used for the detection of K-ras point mutations in lung carcinomas can probably detect a mutation in a sample where the fraction of tumor cells is ≥40%. The
minimal tumor cell fraction necessary for most methods is not well established. For allele-specific oligonucleotide hybridization, this fraction is ~3–10%. To detect those mutations with sequencing, this fraction needs to be 10–25% (33). Therefore, the use of relatively insensitive methods for point mutation detection, which can not discriminate cells containing a mutation in a background of cells with wild-type DNA, could lead to underestimation of positive cases.

Previous studies have already reported that the frequency of K-ras point mutations detected with sensitive methods is increased compared with less sensitive methods of point mutation detection such as allele-specific oligonucleotide hybridization and PCR in combination with denaturing gradient gel electrophoresis (12, 33, 37, 38). Because K-ras mutations can be present in only a subpopulation of the tumor cells (12), a highly sensitive assay is required for the detection of these mutations. In addition, this method should be very specific but not time-consuming, technically complex, or hazardous because of use of radioactive materials. We have previously described a rapid, reliable, and highly sensitive method for detection of known point mutations, Point-EXACCT (35, 36). The purpose of this study was to validate Point-EXACCT for detection of K-ras point mutations in a large series of NSCLC samples. The detection of K-ras point mutations obtained with Point-EXACCT was compared with two different cycle sequencing methods.

Materials and Methods

PATIENTS AND TISSUES

Tumor samples obtained from 89 patients consecutively treated with lobectomy or pneumectomy for NSCLC in the Maastricht University, Maastricht, and De Wever Hospital, Heerlen, were evaluated for point mutations in codon 12 of the K-ras gene. Fresh tissue blocks were stored at −70 °C after surgical resection. The percentage of tumor cells of each frozen tumor sample was subjectively estimated on hematoxylin and eosin-stained sections. Histopathologic classification was performed according to the World Health Organization guidelines (39) on formalin-fixed, paraffin-embedded, and subsequently hematoxylin and eosin-stained slides. DNA was isolated from 25-μm sections of tissue blocks, using a rapid lysis procedure (36). The study protocol was approved by the local ethical committee.

CONTROLS AND RECONSTRUCTION EXPERIMENTS

Purified DNA from several cell lines with known K-ras codon 12 alterations was used for positive controls in the Point-EXACCT method. The following cell lines were utilized: HL60 and NCI-H716, which have the wild-type DNA for K-ras codon 12 (1, 40); A549, which has a homozygous serine mutation (AGT) (41); and Calu-1, which has a heterozygous cysteine mutation (TGT) (42). SW480 is homozygous for the K-ras codon 12 valine mutation (GTT) (42).

To demonstrate the sensitivity of Point-EXACCT, several reconstruction experiments were made in which vital cells bearing a K-ras codon 12 mutation were serially diluted in wild-type cells as described before (36). Reconstruction experiments for codon 12 bases 1 and 2 were performed with cell lines A549 and Calu-1, and SW480, respectively.

K-ras Amplification and Point Mutation Detection

The conditions for K-ras amplification and point mutation detection by fluorescent cycle sequencing and Point-EXACCT have been described in detail previously (36). Briefly, amplification for double-stranded fluorescent cycle sequencing and Point-EXACCT was performed with primers of exon 1 of the K-ras gene outside the codon 12 region to generate an amplification product of 204 bp. All samples were subjected in duplicate to both amplification and point mutation analysis by Point-EXACCT. Point-EXACCT was performed with probes directed against bases 1 and 2 of K-ras codon 12. An adaption of the Point-EXACCT method was made, using 1.6 pmol of both biotinylated capture probes and digoxigenin-labeled detection probe for base 2 of K-ras codon 12. In addition, ligation reactions for base 2 of K-ras codon 12 were performed in 50 μL of 1× ligation buffer (Promega Corporation), consisting of 30 mmol/L Tris-HCl, pH 7.8, 10 mmol/L MgCl2, 10 mmol/L dithiothreitol, and 0.5 mmol/L ATP, without addition of formamide.

K-ras amplification for single-stranded fluorescent cycle sequencing was performed in a 233-bp product, using a secondary PCR approach with the same thermal cycling profile as described before (36).

For fluorescent cycle sequencing, amplification products were digested with exonuclease I (United States Biochemical) and shrimp alkaline phosphatase (United States Biochemical) to remove excess oligonucleotide primers and deoxynucleoside triphosphates. For single-stranded fluorescent cycle sequencing, the products were made single-stranded after cleanup of the amplification products (36). Cycle sequencing was performed on a Perkin-Elmer 9600 thermal cycler (Perkin-Elmer) with the Taq Dye Deoxy™ terminator cycle sequencing kit (ABI, Perkin-Elmer) with the sequencing primer K18 (36). Sequencing reactions were analyzed on an ABI 377 sequencer according to the manufacturer’s instructions. The three mutation detection procedures were performed in a blinded fashion.

Results

CLINICAL DATA

Tumor samples of 89 patients, 71 men and 18 women treated with lobectomy or pneumectomy for NSCLC, were investigated. The mean age and standard deviation for men was 65 ± 9 years and for women was 58 ± 13 years. The distribution of non-small cell lung cancer by histological type was 52 squamous cell carcinomas; 29
adenocarcinomas, including one adenocarcinoma with neuroendocrine characteristics and two bronchoalveolar carcinomas; and 8 large cell carcinomas.

The mean and standard deviation of the percentage of viable tumor cells, normal tissue components, and necrotic cells were determined in hematoxylin and eosin-stained frozen sections of the tissue block. For the tumor part and normal components, these values were 45% ± 15% (range, 5–80%) and 37% ± 14% (range, 10–90%), respectively. Furthermore, for the necrotic cells, these values were estimated to be 18% ± 19% (range, 0–90%).

**POINT-EXACCT**

On all 89 NSCLC cases, the Point-EXACCT procedure was performed for K-ras codon 12 point mutation analysis. Positivity levels were based both on positive and negative controls for K-ras codon 12, bases 1 and 2 separately. As an external positive control for the sensitivity of the Point-EXACCT method, DNA originating from a cell mixture with a known dilution of 1 cell with a mutation in 15 000 wild-type cells was used in each experiment. All samples were separately tested with each of four different base-specific probes (G, A, T, and C). As a positive external control for cells with a mutation for base 1 of K-ras codon 12, the cell lines A549 and Calu-1 were used, which gave positive relative absorbance values for the specific mutation after hybridization and ligation with probes A and T, respectively. For base 2 of codon 12, two different dilutions of cell line SW480 in the wild-type cell line HL60 (1:3000 and 1:15 000) represent positive controls, leading to positive signals for the specific mutation after hybridization and ligation with probe T.

As an external negative control for base 1, relative absorbance values of negative probes (background values) T and C and A and C were used for the cell lines A549 and Calu-1, respectively. For base 2 of codon 12, the values for probes A and C on the SW480 mixture gave background values. These data were taken to calculate the mean, standard deviation, and threshold values for each specific nucleotide, bases 1 and 2 separately. Overall, the threshold value based on negative control probes for each specific nucleotide was calculated by the mean plus 3 standard deviations of the background values. Thus, two different threshold levels can be set in this quantitative assay: one based on the negative control and the other on the positive control.

A sample was called positive for a mutation of bases 1 and 2 in the Point-EXACCT assay when the duplicate signals for a specific nucleotide exceeded the threshold value for the corresponding nucleotide, based on the negative control as well as, if available, the threshold value for the positive control.

The results of K-ras codon 12 point mutation analysis obtained with Point-EXACCT revealed an overall mutational frequency of 21 (24%) of 89 in the NSCLC samples. Table 1 shows the frequency of K-ras codon 12 mutations in the total group of 89 NSCLC, represented by histological type, and further summarizes the type of mutations, identified with Point-EXACCT, resulting in different amino acids. Four mutations were G-to-A transitions, which gave rise to two AGT (serine) and two GAT (aspartic acid). The majority of mutations were G-to-T transversions, including 3 TGT (cysteine) and 12 GTT (valine). Two double mutations were found, in which the wild-type sequence GGT was converted to ATT (isoleucine).

Because Point-EXACCT is a colorimetric assay, a level of positivity can be discerned. Relative absorbance signals similar to or higher than 30%, obtained from duplicate measurements with Point-EXACCT, were called high fraction mutations. On the basis of the relative absorbance signals obtained from several reconstruction experiments (36), this 30% threshold signal corresponded to a mutation fraction between 1/625 and 1/3125. Signals below this threshold were further classified as low fraction mutations.

K-ras codon 12 mutations revealed by Point-EXACCT showed high fraction mutations in 9 patients, and 12 patients were found to harbor low fraction mutations. Although high fraction mutations were mostly found in patients with adenocarcinoma, the majority of low fraction mutations were demonstrated in patients with squamous cell carcinomas (see Table 2).

**SEQUENCING OF DOUBLE-STRANDED AMPLIFICATION PRODUCTS**

Amplification products for K-ras were utilized as templates for direct cycle sequencing with the use of Dye Deoxy terminators. This assay was successful on 81 lung carcinomas. Only the wild-type sequence for K-ras codon 12 could be detected, indicating low sample sensitivity. Sequencing reactions of the remaining eight cases, including four cases that were positive by Point-EXACCT, failed because of insufficient DNA.

<table>
<thead>
<tr>
<th>Table 1. Distribution of K-ras mutations found with Point-EXACCT by histological subtype.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Histology</strong></td>
</tr>
<tr>
<td>SQCC</td>
</tr>
<tr>
<td>Adeno</td>
</tr>
<tr>
<td>LCC</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>

* ATT represents a double mutation.
* SQCC, squamous cell carcinoma; Adeno, adenocarcinoma; and LCC, large cell carcinoma.
The use of T7 gene 6 exonuclease yields highly efficient data for sequencing (36). With single-stranded amplification products as templates for the direct cycle sequencing method, 80 of 89 NSCLC were examined. The remaining nine cases, including four cases positive by Point-EXACCT, could not be analyzed, also because of lack of DNA. Exonuclease-enhanced sequencing detected six NSCLC cases positive for K-ras codon 12. K-ras alterations detected with exonuclease-enhanced sequencing consisted of four G-to-T transversions, including three TGT (cysteine) and one GTT (valine) and two G-to-A transitions, which both produced AGT (serine). Those six mutations were also demonstrated with Point-EXACCT, which showed highly positive signals for the specific mutation, indicating high fraction mutations. The remaining three cases that had been detected as high fraction mutations with Point-EXACCT were not analyzed by either sequencing methods. Furthermore, cases with low fraction mutations identified with Point-EXACCT could not be detected with either double-stranded or single-stranded sequencing. For one patient with a low fraction mutation demonstrated with Point-EXACCT, no DNA was available for either sequencing methods. Thus, analysis with Point-EXACCT revealed K-ras mutations in 11 lung carcinomas that were not detected by single-stranded cycle sequencing. This can be explained by the high sensitivity of Point-EXACCT. With regard to the spectrum of K-ras alterations between different methods, exactly the same type of mutation demonstrated with single-stranded sequencing was identified with Point-EXACCT. This validates point mutation analysis by Point-EXACCT.

Discussion

This study validates the Point-EXACCT method for the detection of known point mutations in a large group of patients with NSCLC by comparing the results with direct cycle sequencing of double- and single-stranded amplification products.

When double-stranded amplification products were used as templates for fluorescent cycle sequencing, no K-ras codon 12 alterations could be demonstrated. In other words, only the nucleotide sequence of the majority of cells (wild-type) could be detected, because the discrimination between cells containing a mutation and wild-type cells is not well achieved by fluorescent cycle sequencing of double-stranded amplification products.

We improved the sensitivity of detection by using single-stranded amplification products as templates for fluorescent sequencing. This was established by exonuclease digestion of amplification products before direct cycle sequencing, producing increased hybridization efficiency of the sequencing primer to the single-stranded amplification products, as reported previously (36). The results after exonuclease digestion demonstrated that all cases with a mutation identified by single-stranded sequencing were also positive for Point-EXACCT. No discordanes in the types of mutations were seen between different methods. No cases were found positive by sequencing and negative with Point-EXACCT. Remarkably, additional cases were identified with Point-EXACCT. This confirms the accuracy and high sensitivity of Point-EXACCT and validates use of the Point-EXACCT method for point mutation detection in lung carcinomas.

Point mutation analysis in K-ras codon 12 by Point-EXACCT detected K-ras point mutations in 15% of squamous cell carcinomas, 35% of adenocarcinomas, and 38% of large cell carcinomas. Furthermore, one patient with a large cell carcinoma and one patient with an adenocarcinoma showed a similar double mutation. From two patients with bronchoalveolar carcinomas, one was positive for a point mutation in K-ras codon 12.

Our results obtained with Point-EXACCT support previous findings suggesting that K-ras mutations are not restricted to only adenocarcinomas (14, 43, 44). The 35% frequency of K-ras mutations in adenocarcinomas coincides with several other reports (14, 43), but a much higher frequency was found in our study for large cell carcinomas, compared with others (9, 43). Furthermore, the 15% frequency of cases positive in squamous cell carcinomas is the same as found in several other studies (38, 43), but is higher than first reports on K-ras, which used less sensitive methods that showed no K-ras point mutations in this histological subtype (3, 9).

Among different base substitutions, a predominance of G-to-T transversions, which changed wild-type glycine to valine in most of the cases, was found. The predominant occurrence of G-to-T transversions is in agreement with previous studies, in which the wild-type glycine was changed most often to valine (4, 10, 24, 38, 45).

With regard to the fact that low fraction mutations revealed by Point-EXACCT do not always correspond to a low percentage of tumor cells within these tumors, this implies that only subpopulations of the tumor are positive for K-ras codon 12. This was in agreement with results obtained by other groups (12, 38, 46), who reported a substantial number of tumors containing K-ras point mutations only in a small fraction of the tumor. Those mutations were predominantly found in patients with squamous cell carcinoma. We made a separation between

### Table 2. Differences in high and low fraction mutations found in different histological subtypes by Point-EXACCT.

<table>
<thead>
<tr>
<th>Histology</th>
<th>High fraction</th>
<th>Low fraction</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>SQCC</td>
<td>1</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Adeno</td>
<td>6</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>LCC</td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>9</td>
<td>12</td>
<td>21</td>
</tr>
</tbody>
</table>

* SQCC, squamous cell carcinoma; Adeno, adenocarcinoma; and LCC, large cell carcinoma.

* One patient with an adenocarcinoma and another patient with a LCC contained one double mutation, both of which indicated high fraction mutations.
cases containing high fraction mutations or low fraction mutations. Also in our study, the majority of low fraction mutations were found in patients with squamous cell carcinoma, whereas high fraction mutations were identified more often in patients with adenocarcinoma.

In line with the clonal expansion theory of tumor formation, the mutation would be found in a high fraction of cells if this event occurs early in this process. This is probably the case for the mutations found in patients with adenocarcinoma. Because only a small population of tumor cells contain the specific K-ras alterations in the majority patients with squamous cell carcinoma, this would suggest that those mutations are not an early event but rather may occur in a later subclone of the tumor, at the tumor progression state.

Although the detection of K-ras point mutations was improved by the use of single-stranded amplification products as templates for direct cycle sequencing, there is still a risk for underestimation of cases positive for K-ras codon 12 point mutations, using direct cycle sequencing, because a relevant number of mutations were apparently below the detection limit of those methods. This is caused by the occurrence of low fraction mutations in lung carcinomas in combination with the presence of a mixture of cells with wild-type DNA in clinical tissue samples.

Point-EXACCT has several advantages, compared with previously described highly sensitive methods. Keohavong and co-workers (38, 47) reported detection of low fraction mutations, using a combination of restriction enzyme digestion and denaturing gradient gel electrophoresis. This assay includes several rounds of amplification and restriction enzyme digestion, and for visualization of the products, polyacrylamide gel electrophoresis is required. Because this assay uses several amplification rounds, a number of false positive cases were demonstrated because of Taq-induced AGT and GAT mutations at a mutant fraction between 10^{-3} and 10^{-4}. Mills et al. (33) reported a highly sensitive assay to detect 1 mutant K-ras allele between 10^6 alleles in patients with leukemia. Although the sensitivity of this method is extremely high, this method is time-consuming and very cumbersome, using two to three amplification and restriction enzyme steps. Furthermore, the high number of amplification cycles increases the risk of false positivity by extension of base mispairs by Taq DNA polymerase (48, 49). However, this risk may be reduced by using other polymerases with greater fidelity (50). An adaption of the method described by Mills et al. (12), being less sensitive, reported increased prevalence of K-ras mutations in lung adenocarcinoma. Because Point-EXACCT uses only one amplification round, the risk of false positivity possibly due to numerous amplification cycles and PCR product handling is dramatically diminished. This was further demonstrated by the fact that identical results were obtained in each duplicate sample, making false positivity highly unlikely.

In conclusion, using a large series of non-small cell lung cancers for the analysis of mutations occurring at either a high or low mutant fraction in K-ras codon 12, we have demonstrated an increased number of mutations found with Point-EXACCT compared with traditional methods such as sequencing. Because K-ras point mutations are found to occur early during lung cancer development, the high sensitivity of Point-EXACCT makes it a useful method for the detection of K-ras in a heterogeneous cell population, where only a small fraction of tumor cells containing a mutation are present among much larger numbers of cells with wild-type DNA. This method is now being used for detection of K-ras point mutations in sputum samples, in which it has been shown to discern the presence of a minority or a single mutant cell among an excess of cells with wild-type DNA (manuscript in preparation). These findings could enhance the potential for clinical utility of the genetic alterations such as K-ras.

This study was funded in part by the Scientific Committee of Smoking and Health, The Netherlands.

References

12. Mills NE, Fishman CL, Rom WM, Dubin N, Jacobson DR. Increased
prevalence of K-ras oncogene mutations in lung adenocarcinoma.


