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Additional value of *K-ras* point mutations in bronchial wash fluids for diagnosis of peripheral lung tumours

V.A.M.C. Somers*, A.M.J. van Henten**, G.P.M. ten Velde**, J-W. Arends*, F.B.J.M. Thunnissen*

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ABSTRACT: The purpose of this study was to examine the additional diagnostic value of *K-ras* point mutations in the clinical diagnosis of peripheral lung tumours.

To this end, bronchial wash fluids obtained during bronchoscopy from patients suspected of having lung cancer were studied. Only those patients were investigated for whom the cytological diagnosis was not conclusive for malignancy. As a control group, patients without lung cancer were investigated. The method of "point mutation detection using the exonuclease amplification coupled capture technique" (Point-EXACCT) for analysis of *K-ras* codon 12 was performed in bronchial wash fluids and the corresponding tumour tissue, if available.

***K-ras* point mutations were identified in 4 out of 19 (21%) bronchial wash fluids from patients without a decisive diagnosis of malignancy. The diagnosis of malignancy was further based on cytological examination of bronchial brush specimens, perthoracic needle aspiration, histological investigation of biopsy and resection specimens, needle aspiration of a lymph node in the neck and pleural fluid examination. Four of the patients who were *K-ras*-positive yielded positive malignant tissue *via* bronchoscopy even though the bronchial wash was negative for malignancy. The bronchial wash was positive for *K-ras* in two of the four patients whose tumour tissue demonstrated the *K-ras* mutations. Analysis of bronchial wash fluids from 11 patients without lung cancer revealed no *K-ras* codon 12 mutations.**

In conclusion, *K-ras* point mutations can be identified in bronchial wash fluids obtained during bronchoscopic procedures. *K-ras* can be used as a biomarker in the clinical diagnosis of lung cancer and may serve as an adjunct to cytology in lung cancer diagnosis.

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Depts of *Pathology and **Pulmonology, Maastricht University, Maastricht, the Netherlands.

Correspondence: V.A.M.C. Somers
Maastricht University
Dept of Pathology
P.O. Box 5800
6202 AZ Maastricht
The Netherlands
Fax: 31 433876613

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Lung cancer is a common and deadly disease. At the time of diagnosis, lung cancer is often locally or systemically advanced. Therefore, only approximately 25% of all patients with lung cancer present with locally defined tumours which are considered amenable to surgical resection [1].

Patients being evaluated for suspected lung cancer based on the presence of a lung mass on chest radiograph often receive diagnostic fibreoptic bronchoscopy during which a biopsy is taken for tissue diagnosis and additional bronchial brush and wash fluid sampling is performed for cytological examination. It is generally accepted that, in the absence of a histological diagnosis, treatment for lung cancer can be based on the cytological diagnosis of malignancy in bronchial brush or wash specimens. In the case of a peripheral lung tumour, which is inaccessible for biopsy, cytological examination of wash specimens may be the only diagnostic modality. However, cytological diagnosis is sometimes equivocal, especially in specimens in which only a limited number of tumour cells are present between several alveolar macrophages and other nonmalignant cells.

Over the past decade, remarkable advances have been made in understanding lung cancer biology as well as diagnostic technology. A number of molecular abnormalities have been shown to be characteristic of certain lung cancers. Point mutations of the *K-ras* gene are found in 30–50% of adenocarcinomas of the lung and are present in a smaller percentage of squamous cell carcinomas [2–6]. These mutations have also been identified in the bronchoalveolar lavage fluid of patients with suspected lung cancer [7–9].

In a recent study, the highly sensitive method, "point mutation detection using the exonuclease amplification coupled capture technique" (Point-EXACCT) for *K-ras* point mutation detection was successfully applied in the analysis of sputum from patients with adenocarcinoma of the lung [10]. Since with this technique a limited number of cells with a point mutation can be detected within a majority of genetically normal cells, the question arises as to whether this technique can be helpful in determining the nature of a peripheral lung tumour. The identification of these mutations in clinical specimens, which are otherwise not diagnostic for malignancy, may improve the diagnostic yield of the fibreoptic bronchoscopic procedure.

The aim of this study was to examine the additional diagnostic value of *K-ras* point mutations in the analysis of

bronchial wash fluids from patients under suspicion of lung cancer. To this end, a consecutive series of bronchial wash fluids without a malignant cytological diagnosis was investigated using Point-EXACCT in patients with radiographic abnormalities and in a control group.

Materials and methods

Patients

Between January and May 1997, 140 consecutive patients with a lung mass visible on radiography or chronic cough underwent diagnostic fibreoptic bronchoscopy at the University Hospital, Maastricht. Bronchial wash fluids from 19 patients with radiographic abnormalities but no cytological diagnosis of malignancy were used in this molecular study. In addition, bronchial wash fluids from 11 patients with nononcological diseases were used as controls. The routine diagnostic procedure consisted of bronchial brushing and washing for cytological investigation and bronchial biopsy for histological examination. The fibreoptic bronchoscopes were routinely cleaned by means of thermal–chemical disinfection. Firstly, the bronchoscopes were rinsed and swept with lukewarm tap water for 3 min followed by treatment in a Bilimed SLE 2000 (Bilimed, Ballwil, Switzerland). Secondly, they were treated with a detergent (Sekumatic FRE; Henkel Ecolab, Düsseldorf, Germany) for 6 min followed by treatment with a specific disinfectant (Sekumatic glutaral 209 $g \cdot L^{-1}$) (Henkel Ecolab, Germany) combined with deionized water for 6 min. The bronchoscopes were further washed with hot (95°C) deionized water for 5 min and then dried and cooled in a dedicated drying cabinet for $\geq$1 hr at room temperature.

The cytological diagnosis can be categorized into four groups: 1) no malignant cells present; 2) atypia, in which the cytologist notices slight changes but expects these still to be benign; 3) suspicion of malignancy, in which the cytologist finds abnormal cells but a definite diagnosis of malignancy cannot be made. This may be due to a very limited number of tumour cells and/or minimal deviation from the normal range; and 4) malignant cells, for which the cytological diagnosis is certain, bearing in mind the desired 100% specificity for malignancy. In 19 patients with a peripheral tumour, the cytological diagnosis of malignancy was not made on the basis of bronchial wash specimens. These were taken for further molecular study. Molecular analysis was performed without knowing the status of the clinical diagnostic procedure.

Histology

Biopsy specimens were routinely fixed in neutral buffered formaldehyde and embedded in paraffin. From resection specimens of patients with non-small cell lung cancer (NSCLC), frozen tumour specimens were obtained if available and used for isolation of deoxyribonucleic acid (DNA). Biopsy and resection specimens were available from 16 patients. Paraffin sections (5×20 μm) were dewaxed in xylene and rehydrated. DNA was isolated by means of proteinase K digestion [11]. Of 16 biopsy or resection specimens collected, seven were used for DNA isolation and molecular analysis.

Cytology

Bronchial wash fluids were treated according to a standardized protocol [12]. In brief, after local anaesthesia of the upper respiratory tract, the fibreoptic bronchoscope was securely wedged in an airway adjacent to the radiographically defined lesion. The wash fluid was immediately mixed with an excess of alcohol–Carbowax and subsequently transported to the pathology department for further processing of the specimens. Specimens were first put into the blender to break up any mucus. After centrifugation of the specimens for 10 min at $560 \times g$, the pellets were used for preparation of two smears. These were alcohol-fixed and Papanicolaou-stained. The remainder of the specimens were centrifuged for 7 min at $413 \times g$ and the pellets washed three times in 4 mL 96% alcohol. Specimens were then mixed gently and further centrifuged for 7 min at $413 \times g$. The supernatants were discarded and the pellets dissolved in 50–400 μL lysis buffer, depending on the number of cells, for DNA isolation [11].

K-ras *codon 12 point mutation analysis*

Amplification of the first exon of the *K-ras* gene was performed using primers for that gene outside the codon 12 region [11]. The Point-EXACCT method for point mutation analysis of *K-ras* codon 12 was performed as described [11], with probe and corresponding ligase conditions for bases 1 and 2 as previously described [6, 10].

Amplification and point mutation analysis were performed twice for each sample. Samples with signals above the controls in both experiments were deemed positive. Each amplification and point mutation detection experiment included several positive and negative controls.

Results

The Point-EXACCT method was used to identify *K-ras* point mutations present in bronchial epithelial cells from the bronchial wash fluids of 19 patients, 16 males and three females (age 56–82 yrs) under suspicion of having lung cancer. All patients presented with a peripheral lung lesion on chest radiography, but in 10 of them no endobronchial abnormalities were perceived during bronchoscopy. The clinical characteristics of these patients, with corresponding *K-ras* status, are listed in table 1.

Thirteen of the 19 patients had lesions in the right lung, five had lesions in the left lung, and one had a lesion in the neck. The majority of lesions (11 of 19, 58%) were located in the right upper lung.

K-ras codon 12 mutations were found in four of 19 (21%) bronchial wash specimens, and the specific *K-ras* genotypes identified corresponded with two G→A transitions and two G→T transversions.

All four bronchial wash specimens positive for a *K-ras* mutation were found in patients without a decisive cytological diagnosis of malignancy. Only two of the four patients with a *K-ras*-positive bronchial wash specimen were diagnosed with "suspicion of malignancy" on cytological examination, and no malignant cells were found in the other two specimens positive for a *K-ras* mutation. The

Table 1. – Clinical characteristics of patients with corresponding tumour and bronchial wash fluid data

Patient No.	Age yrs	Sex	Smoking status pack-yrs	Tumour site	Tumour size cm	Cytol group*	*K-ras*** wash	Diagnostic procedure****	Time to Diagnosis^{+} days	Final diagnosis	*K-ras*** tumour
1	71	M	35	RU	2.1 × 2.1	1	GGT	Biopsy (3)	143	Adeno	CGT
2	82	M	34	LU	7 × 5.5	1	GGT	Biopsy (3)	44	Adeno	–
3	64	M	90	Lingula	4.0 × 4.0	1	GGT	Brush (1)	–	SCLC	–
4	81	M	60	RU	5.0 × 5.0	3	GGT	Biopsy (1)	–	SqCC	–
5	71	F	50	RMd	8.0 × 8.0	1	GGT	Per. needle (2)	19	Adeno	–
6	63	F	20	RU	1.7 × 1.5	1	GGT	Per. needle (2)	7	Adeno	–
7	56	M	40	Neck	2.0 × 1.5	1	GGT	Needle (2)	13	Meta larynx	–
8	66	M	40	RU	8.0 × 8.0	1	GGT	Resection (3)	42	LCC	GGT
9	77	F	30	LU	2.5 × 1.5	3	GGT	Brush (1)	–	SCLC	–
10	70	M	34	RU	3.0 × 3.0	2/3	AGT	Resection (2)	37	Adeno	AGT
11	58	M	50	RU	4.1 × 4.1	2	TGT	Brush (1)	–	LCC	TGT
12	64	M	30	Lingula	7.0 × 5.5	1	GGT	Brush (2)	27	LCC	–
13	78	M	–	RU	4.5 × 4.5	1	AGT	Pleural fluid (3)	38	Adeno	–
14	71	M	22	RU	7.0 × 5.0	3	TGT	Brush (1)	–	Adeno	–
15	66	M	50	RU	6.0 × 6.0	1	GGT	Brush/biopsy (1)	–	SqCC	GGT
16	59	M	40	RU	4.5 × 4.5	1	GGT	Resection (2)	22	Adeno	AGT
17	62	M	42	RB	2.0 × 2.0	2	GGT	Biopsy (2)	12	LCC	GGT
18	68	M	28	LU	3.0 × 3.0	3	GGT	Brush (1)	–	SCLC	–
19	73	M	55	RU	6.5 × 5.0	1	GGT	Biopsy (4)	34	LCC	–

*: The cytological diagnosis was categorized into four groups: 1, no malignant cells present; 2, atypia (probably benign); 3, suspicion of malignancy; and 4, malignant cells. **: The normal sequence for codon 12 is GGT (glycine). ***: Diagnostic procedure data are presented as the main diagnostic procedure leading to the final diagnosis, with the total number of procedures performed shown in parenthesis. $^{+}$: The time to diagnosis is the time between bronchial washing and diagnosis of malignancy. Cytol.: cytological diagnosis; M: male; F: female; R: right lobe; L: left lobe; U: upper lobe; Md: middle lobe; B: bronchus; Adeno: adenocarcinoma; SCLC: small cell lung carcinoma; SqCC: squamous cell carcinoma; Meta: metastasis; LCC: large cell carcinoma; Brush: bronchial brush specimen; Per. needle: perthoracic needle aspiration; –: no data.

earliest detection of *K-ras*-positive cells in bronchial wash fluid was obtained 38 days before the final diagnosis of malignancy.

Diagnosis of malignancy in the group of 19 patients, was based on cytological examination of bronchial brush specimens in seven patients, perthoracic needle aspiration in two, biopsy specimens in five (including one with a positive, bronchial brush specimen), resection specimens in three, needle aspiration of a lymph node in the neck in one and pleural fluid in one. In only seven patients was the diagnosis of malignancy made on the basis of the first diagnostic bronchoscopic procedure; in the remaining 12 patients, further invasive diagnostic procedures were necessary, which in some cases yielded a diagnosis of malignancy only after several months (table 1). Among the 19 patients with radiologically suspected and histologically or cytologically confirmed carcinomas, the most frequently observed subtype was adenocarcinoma (eight cases).

Of the four patients with a *K-ras*-positive bronchial wash specimen, two had a visible endobronchial tumour. In one of these two patients, the diagnosis of lung cancer was based on the presence of malignant cells in a bronchial brush specimen. In the other, the routine diagnostic procedure was not conclusive. The diagnosis of malignancy was later made on the basis of pleural fluid aspiration. In the two patients without a visible endobronchial tumour, the brush specimen revealed malignant cells in one patient and the clinical diagnosis of malignancy was histologically confirmed after lobectomy in the other.

In seven of 19 patients with lung cancer, histological tumour specimens were examined for the presence of *K-ras* point mutations using Point-EXACCT. Three of the seven cases showed the wild-type sequence GGT for *K-ras* codon 12. In two of the four *K-ras*-positive tumours, the corresponding wash specimens showed the same *K-ras* mutation. In the other two patients with a *K-ras*-positive tumour, the bronchial wash fluid was negative, rendering the presence of tumour cells unlikely. Interestingly, no *K-ras* mutations were found in the bronchial wash fluids corresponding to *K-ras*-negative tumours.

Histological classification of the *K-ras*-positive tumour specimens showed three adenocarcinomas and one large cell carcinoma. The mutations identified in biopsy or tumour specimens from surgical resection were two G→A transitions, which resulted in serine mutations, and one G→T and one G→C transversion, which coded for a cysteine and an alanine mutation, respectively.

As a control group, bronchial wash specimens obtained from 10 patients with chronic bronchitis (1), chronic obstructive pulmonary disease (1), abscess (2), pneumonia (3), persistent infiltrate (2) and interstitial infiltrate (1) and a bronchial biopsy from one patient with tonsil carcinoma were analysed. These patients did not subsequently present with a tumour lesion (14–18 months follow-up). Of the 11 cases analysed, eight were males and three females, with ages ranging 46–74 yrs. On the basis of routine cytological investigation, all 11 cases were classified into category 1, *i.e.* no malignant cells were present. Point mutation analysis of *K-ras* codon 12 using Point-EXACCT did not reveal any mutation.

Discussion

Patients with a peripheral lung mass on radiography under suspicion of having lung carcinoma underwent bronchoscopic procedures, including bronchial biopsy and

brush specimens. The inaccessibility of the affected peripheral region to the bronchoscope frequently causes a failure to yield histological material proving malignancy, and, as a consequence, diagnosis is attempted by means of cytological examination of bronchial wash fluid. Although potentially better sampling of peripheral tumour cells can be obtained *via* bronchial wash specimens, diagnosis of malignancy is sometimes equivocal, especially in cases in which only a very limited number of tumour cells are present in the clinical specimen. In those patients in which a clinical diagnosis was made only subsequent to a *K-ras*-positive bronchial wash specimen, the Point-EXACCT method could offer clinical diagnostic potential for malignancy.

With the use of Point-EXACCT, *K-ras* mutations were identified in bronchial wash specimens from four out of 19 patients under suspicion of having lung cancer, in whom cytological examination was not diagnostic of malignancy. In these four patients, a molecular abnormality supporting the diagnosis of malignancy was present. Although it is realized that, in some of these four patients, the original diagnosis was made by means of cytological examination of the brush specimens obtained during the same bronchoscopic procedure, the outcome of the molecular study strongly suggests that, if all bronchoscopically obtained samples were not conclusive, the additional molecular test may lead to an earlier diagnosis of malignancy and avoid the need for additional invasive diagnostic procedures.

At present, the question is whether or not diagnosis of malignancy can be based on the identification of a *K-ras* mutation in bronchial wash specimens, without cytological proof of malignancy. The present study highlights a number of issues arising from this molecular diagnostic approach to the early diagnosis of malignancy.

With routine cytological investigation, only some of the cells obtained by means of bronchial washing are studied, *i.e.*, in this procedure, diagnosis of malignancy is based on the examination of the limited number of cells present in smears on two slides. A small number of tumour cells and/or minimal deviation from normal may explain the false negative results of the cytological diagnostic procedure. In contrast, the particularly large number of cells studied using Point-EXACCT, *i.e.* ~12,000–15,000 cells·nucleotide^{-1}, makes the discovery of *K-ras*-positive cells more likely. The other explanation for a "false negative" diagnostic test is the absence of tumour cells in the bronchial wash fluid. In that situation, cytology as well as Point-EXACCT will not lead to the diagnosis of malignancy.

K-ras point mutations occur in approximately 50% of adenocarcinomas, which represent the majority of peripheral lung carcinomas. Squamous cell carcinomas and small cell lung carcinomas (SCLCs) are usually centrally located. For these tumour types, the additional value of *K-ras* gene analysis is limited. This observation is consistent with the results of other studies, which indicate no *K-ras* mutations in SCLC subtypes and only a few in squamous cell carcinomas [13–15]. However, the centrally located tumours have a higher chance of being visible *via* bronchoscopy and being diagnosed by means of brush specimen and/or biopsy. Importantly, by investigating bronchial wash fluids for *p53* mutations, *e.g.* in patients suspected of having SCLC, the sensitivity of detection could be considerably improved [16, 17].

In determining *K-ras* in bronchial wash specimens for diagnostic purposes, the question arises as to whether a positive test, *i.e.* the presence of a *K-ras* point mutation, is always associated with lung cancer. In the case of a peripheral lesion on the radiograph of a patient >40 yrs of age, the probability of lung carcinoma is high, especially if there is a history of smoking. The probability of benignancy of a peripheral lung tumour is estimated on the basis of published data. The probability of a lesion on the radiograph being benign (coin) is estimated at 40% [18, 19]. In theory, a situation may exist in which the Point-EXACCT method is positive for a *K-ras* mutation and the lesion is benign: *e.g.* a hamartoma with adjacent *K-ras*-positive atypical alveolar hyperplasia (AAH). An approximation of the probability of this occurrence can be obtained by multiplication of the individual probabilities (benign lesion, 40% [18, 19] × (incidence of AAH in resection specimens of all cancers, 5% [20]) × (*K-ras*-positivity in AAH, 25% [21])=0.5%). This probability is similar to the false positive rate of cytology. Therefore, curative surgery on the basis of a shadow on a radiograph plus a *K-ras*-positive Point-EXACCT test is reasonable in the absence of conclusive routine diagnostic procedure results.

These days, highly sensitive molecular techniques are used for the detection of molecular alterations in various excretions/secretions. These specimens often contain the same genetic alteration as the tumour. The presence of genetic alterations has been demonstrated in various body fluids, *e.g.* the effluent samples from patients who had undergone colonoscopy and yet were free of colorectal disease at the time of examination [22], faecal material from patients with colorectal cancer [23, 24], pancreatic juice from patients with pancreatic cancer [25, 26] and urine obtained on surgery of patients with bladder cancer [27].

In previous reports, the use of *K-ras* mutations in bronchoalveolar lavage fluids and pleural fluids as a clinical marker for lung cancer diagnosis has been described [7–9]. In this study, *K-ras* codon 12 point mutations were found in the bronchial wash fluids of patients suspected of having lung cancer but not in those of a series of patients without pulmonary malignancy. These data demonstrate the high specificity of the Point-EXACCT method and further affirm the use of *K-ras* mutations as a marker in the clinical diagnosis of peripheral lung tumours. In theory, contamination might occur due to cell/DNA transfer *via* the fibreoptic bronchoscope, *i.e.* cells from one patient might be added to material from another patient. Importantly, the European guidelines for fibreoptic bronchoscope disinfection include a step involving glutaraldehyde, which cross-reacts with DNA and impairs amplification, rendering the possibility of false positivity due to contamination highly unlikely. The present data are consistent with the results of MILLS *et al.* [7], who did not find any *K-ras* mutations in specimens with a diagnosis other than NSCLC. Contradictory results were obtained in the study of YAKUBOVSKAYA *et al.* [24] who found a 12.5% frequency of *K-ras* mutations in patients with nononcological disease. In that study, the lower specificity could possibly be improved by inclusion of specific controls during point mutation analysis.

In conclusion, the present study demonstrates that it is possible to detect *K-ras* point mutations in the bronchial

wash fluids of peripheral tumours. Molecular analysis can serve as an important adjunct to bronchoscopy in lung cancer diagnosis.

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