The role of K-ras point mutation detection in lung cancer : towards a strategy for early detection

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The role of K-ras point mutation detection
in lung cancer :
Towards a strategy for early detection

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The role of K-ras point mutation detection
in lung cancer :
Towards a strategy for early detection

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Veerle Anne-Marie Christine Somers

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Promotor :

Prof. Dr. J.W. Arends

Co-promotor :

Dr. F.B.J.M. Thunnissen

Beoordelingscommissie :

Prof. Dr. E.F.M. Wouters (voorzitter)

Prof. Dr. P.E. Postmus (Vrije Universiteit Amsterdam)

Prof. Dr. M.F. von Meyenfeldt

Prof. Dr. F.C.S. Ramaekers

Prof. Dr. J.K. Field (Roy Castle International Centre for Lung Cancer Research, Liverpool, UK)

Aan mijn ouders

Contents

Abbreviations 9

Chapter One. General Introduction 11

1. Introduction 12

1.1 Epidemiology

1.2 Smoking

1.3 Clinical characteristics

2. Genetic alterations in lung cancer 13

2.1 Genetic alterations in carcinoma of the lung 14

2.1.1 K-ras point mutation detection in lung carcinomas 16

2.2 Genetic alterations in premalignant lesions 19

2.3 Time-point of genetic alterations 20

2.3.1 Field cancerization and chemoprevention 21

3. Lung cancer screening 22

3.1 Primary prevention 22

3.2 Results of screening studies for lung cancer 22

3.3 Genetic alterations as biomarkers in lung cancer 25

4. Aims of the present study 26

Chapter Two. Development of Point-EXACCT 27

Chapter Three. Sensitivity of Point-EXACCT 33

Chapter Four. Validation of Point-EXACCT 51

Chapter Five. K-ras detection in sputum 61

Chapter Six. K-ras in non-malignant bronchial epithelium 75

Chapter Seven. K-ras in bronchial wash fluids 87

Chapter Eight. K-ras and prognosis 97

Chapter Nine. Epilogue 115

1. Introduction 116

2. Technical aspects 116

2.1 Development of Point-EXACCT 116

2.2 Sensitivity of Point-EXACCT 118

2.3 Threshold values 118

2.4 Technical improvements of Point-EXACCT 119

3. The role of K-ras point mutation detection in NSCLC with Point-EXACCT 120

4.	Suggestions for further research towards early detection	121
4.1	Identification of high-risk groups	121
4.2	Molecular diagnostic techniques	122
4.3	A new array of diagnostic imaging techniques	122
4.4	Towards a strategy for early detection	123
References		125
Chapter Ten. Summary/Samenvatting		143
Dankwoord		151
Curriculum Vitae		155
List of publications		157

Abbreviations

A	adenine
AAH	atypical alveolar hyperplasia
adeno	adenocarcinoma
AGT	serine (ser)
ATP	adenosine triphosphate
ATT	isoleucine (ile)
Bio.	biotin
bp	base pair
BSA	bovine serum albumin
C	cytosine
Cdc25	cyclin dependent cyclase 25
CDKN2	cyclin dependent kinase/p16/MTS1
CGT	arginine (arg)
CI	confidence interval
CIS	carcinoma in situ
c-Kit	tyrosine-kinase receptor
COPD	chronic obstructive pulmonary disease
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dITP	deoxyinosine triphosphate
DMEM	Dulbecco's modified Eagle's medium
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
erb B2	human epidermal growth factor receptor/Her-2/neu
EXO	exonuclease
Dig.	digoxigenin
EXACCT	exonuclease-amplification coupled capture technique
FHIT	fragile histidine triad
G	guanine
GAPs	GTPase-activating proteins
GAT	aspartic acid (asp)
GCT	alanine (ala)
GDP	guanosine diphosphate
GGT	glycine (gly)
GTP	guanosine triphosphate
GTT	valine (val)
H-ras	Harvey ras
Her-2/neu	human epidermal growth factor receptor
hnRNP	heterogeneous nuclear ribonucleoprotein

K-ras	Kirsten ras
LCC	large cell carcinoma
LCR	ligase chain reaction
LOH	loss of heterozygosity
min	minutes
MTS1	multiple tumor suppressor 1/p16/CDKN2
Mut.	mutation
NF	neurofibromatosis
N-ras	Neuroblastoma ras
NSCLC	non-small cell lung cancer
PALGA	Dutch national pathology-coding computer network
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PCR-PIREMA	PCR-primer-introduced restriction analysis with enrichment of mutant alleles
per. needle	perthoracic needle
POD	peroxidase
Point-EXACCT	point mutation detection using exonuclease-amplification coupled capture technique
Prad	cyclin D1
pStage	pathological stage
p16	protein 16
p21	protein 21
p53	protein 53
rc	regression coefficient
RB	retinoblastoma
SAP	shrimp alkaline phosphatase
SCLC	small cell lung cancer
SD	standard deviation
sec	seconds
SE	standard error
SEER	US Surveillance, Epidemiology, and End Results
Sos	son of sevenless
SqCC	squamous cell carcinoma
SSCP	single-strand conformation polymorphism
SSS	phosphorothioate linkages
T	thymine
TGT	cysteine (cys)
TMB	3,3',5,5'-tetramethyl-benzidine dihydrochloride
TNM	tumor-node-metastasis
U	unit
W.T.	wild type

One

General Introduction

1. Introduction

1.1 Epidemiology

Lung cancer is the most commonly occurring malignancy in Europe and the United States and still continues to show an increasing incidence. Although age-adjusted incidence of lung cancer in males reached plateau in 1990 and then started to decline, the pattern in women was that of a significant increase in the same period^{1,2}. The US Surveillance, Epidemiology, and End Results (SEER) lung cancer incidence trends from 1973 to 1990 demonstrated a percentage increase for women (110.5%) that was substantially greater than that for men (9.2%)³. Between 1990 and 1994, the lung cancer mortality rate in women increased about 1.7% per year, but now appears to be leveling off and will presumably decline, reflecting a later peaking of cigarette use^{1,4-7}.

1.2 Smoking

Epidemiologic and experimental data have unequivocally established tobacco smoking as the primary risk of lung cancer, a fact recognized since the first reports appeared on this issue in the 1950s^{2,8-11}. The risk is related to the number of cigarettes smoked, age at starting smoking, and the duration of smoking^{10,12}. Apart from an abundance of epidemiologic data fulfilling the criteria for a causal association, in recent years molecular studies have started to unravel the steps of smoke induced carcinogenesis¹³⁻¹⁷.

It is estimated that smoking accounts for 80-90% of all cases of lung cancer. The vast majority of cases could be prevented by never smoking, and the risk of developing lung cancer could be reduced by smoking cessation. Current trends in cigarette smoking among teenagers show an increase in smoking rates in the Netherlands, emphasizing the continued importance of lung cancer, i.e. the primary prevention as well as the expected high incidence.

The lung cancer incidence and mortality pattern follow after a latency interval of 20 or more years the temporal patterns of cigarette smoking. Estimations of the fraction of lung cancer mortality attributable to smoking for different countries have been made by the International Agency for Research on Cancer using an index of «smoking maturity», based on the extent of past smoking practices in individual nations. Among six major industrialized nations, the United States and England and Wales had the highest indices of smoking maturity, with identical smoking attributable fractions for lung cancer

of 92% in men and 78% in women. Japan had the lowest, with smoking attributable fractions of 53% in men and 36% in women¹⁸.

1.3 Clinical characteristics

Lung cancer is a disease with heterogeneity in clinical behavior, in biology and morphology. This disease is categorized into two major groups: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). SCLC accounts for only 20 to 25% of all lung cancer cases. The remaining 75 to 80% fall into the following histologic types of NSCLC including squamous cell carcinoma (SqCC), adenocarcinoma and large cell carcinoma (LCC). SqCC is thought to arise from the metaplastic and subsequently dysplastic epithelium of the larger, proximal airways. Adenocarcinoma seems to develop in the epithelium of peripheral airways and alveoli. While the histologic definition of LCC is clear (carcinoma in the lung without the characteristics of SCLC, SqCC or adenocarcinoma), in practice some interobserver variability may exist¹⁹. Therefore LCC may include some different types of NSCLC.

Lung carcinomas are divided for treatment purposes in two different groups with different clinical characteristics: SCLC is an aggressive type of tumor that is nearly always metastasized at time of clinical presentation. This type of tumor is treated with chemotherapy or radiotherapy. However, when no metastases are present, SCLC patients undergo lobectomy or pneumectomy. For NSCLC, stage of disease at time of clinical presentation determines the choice of therapeutic intervention. The only approach that offers a potential for cure is surgical resection in localized disease (stage I and II), which occurs in $\pm 25\%$ of all lung carcinomas²⁰. Alternatively, radiotherapy or chemotherapy can be given²¹.

The prognosis of patients with lung cancer is primarily dependent on the stage of the tumor at time of clinical diagnosis. The prognosis for SCLC is generally poor: the two year survival rate is less than 10%. For NSCLC cases, the five year survival rate for stage I tumors is 60-85%, 30-50% for stage II tumors and 0-10% for stage III and IV tumors²²⁻²⁴.

2. Genetic alterations in lung cancer

In general, the initiation and progression of tumors are related to a series of genetic alterations. The process of carcinogenesis involves the accumulation of genetic alterations which can provide growth advantage for a cell containing the mutations. These mutations are either inborn (familial), which are rare in lung carcinogenesis or acquired, the latter presenting more than 99% of the cases. Mutational events in several types of genes seem to underlie this process. These mutations presumably arise from the initiating action of a carcinogen and persist in the genome due to failure of repair prior to cell division. The acquired mutations may be due to i) direct damage to

DNA; e.g. benzo(a)pyrene forms preferential adducts in lung cancer leading to mutational hotspots in the p53 gene²⁵, ii) oxidative stress with subsequent DNA damage; e.g. C-T transitions via 5'-methyl cytosine or iii) other physico-chemical pathways leading to DNA damage. These genetic alterations can occur to a various extent in a number of different cells. At a certain point in time in one of these cells the eventual accumulation of genetic changes leads to a malignant cell with the functional capability of uninhibited growth and spread. A number of animal studies have demonstrated the use of chemical carcinogens inducing molecular alterations in lung carcinogenesis²⁶⁻⁴⁶.

The two major classes of genes that are altered during the development of a malignant tumor are proto-oncogenes and tumor suppressor genes. These genes normally regulate cell growth and differentiation. Proto-oncogenes stimulate cell growth, cell survival or they inhibit apoptosis (programmed cell death). Proto-oncogenes can be activated to become oncogenes by several mechanisms, including mutation, and amplification or overexpression of the normal cellular gene (proto-oncogene).

The second class of genes mutated during carcinogenesis are the tumor suppressor genes, which are involved through mutational inactivation of one copy during somatic tumor development followed by another mutation or loss of the second allele⁴⁷. When these genes become inactivated the function of the proteins that are encoded by the genes alters. This can lead to dysregulation of the cell cycle, such as uninhibited growth or inhibition of apoptosis.

2.1 Genetic alterations in carcinoma of the lung

In lung cancer, a variety of characteristic genetic alterations have been identified. These alterations can be large such as deletions and translocations, while the majority of alterations occurring during the process of lung cancer refer to small alterations, including point mutations and small deletions. Concerning gross alterations occurring in the process of lung tumorigenesis, including large deletions and translocations, no consistent translocations have been reported⁴⁸.

Between both categories of SCLC and NSCLC a number of consistent molecular genetic differences are found. However, there is evidence that both subtypes rely on several common genetic alterations. The genetic alterations occurring in lung carcinogenesis are only partly known yet. Chromosomal deletions and corresponding tumor suppressor gene inactivation in lung cancer are presented in table 1. Differences of specific types of genetic alterations occurring between SCLC and NSCLC are also represented.

The most consistent cytogenetic abnormality in lung cancer is loss of genetic material on the short arm of chromosome 3⁴⁹⁻⁵³. Although many candidate tumor suppressor genes that map to the consistently deleted

segmented regions of chromosome 3 have been described^{48,54-57}, recently attention was focused on the role of the FHIT gene in lung carcinogenesis⁵⁴. The same holds for deletions of loci on chromosome 5q, 8p, 10q and 11q^{58,59}. Frequent loss of heterozygosity at chromosome 9p has been described, involving the gene CDKN2, also known as multiple tumor suppressor 1 (MTS1) or p16^{INK4} 48,60-67.

On chromosome 13q the retinoblastoma (RB) gene is identified which is mutated in the majority of SCLC and to a lesser extent in NSCLC^{48,68,69}. This is also true for the p53 gene, located on chromosome 17p, demonstrating more deletions in SCLC than NSCLC⁷⁰.

SCLC exhibits infrequent loss of 9p, but more losses of 3p, 5q, 13q, and 17p as compared with NSCLC⁷¹.

Table 1. Tumor suppressor gene abnormalities in lung cancer

Tumor suppressor		Abnormal expression frequency	
Genes	Mutation	NSCLC (%)	SCLC (%)
candidates on 3p	deletion	50	90
p16	protein expression	60	—
RB	deletion, altered protein		
	expression, phosphorylation	~20	~100
p53	deletion, point mutation,		
	overexpression	~60	~90

Sources:^{48,72-77}

Major differences in both categories of lung cancer are represented by oncogene mutations and overexpression. Oncogene mutations and overexpression in lung cancer are summarized in table 2. A consistent difference between SCLC and NSCLC is the presence of K-ras point mutations. Those are not detected in SCLC, but are frequently mutated in NSCLC^{78,82}. The NSCLC cells have aberrant expression of another family of dominant proto-oncogenes, the erb B family, whose members include erb B1 and erb B2 (also known as Her-2/neu)^{74,83,84}.

Another family of dominant oncogenes, the myc family, has been found to be abnormally expressed in SCLC cells. The general mechanism of activation of myc in lung cancer is gene amplification with resulting overexpression. Unlike ras and erb B, myc proto-oncogenes are frequently aberrantly expressed in SCLC cells and only rarely in NSCLC cells^{72,85-87}.

Cyclin D, which activates cyclin dependent kinases is frequently overexpressed in NSCLC, leading to inactivation of Rb, while c-Kit, a tyrosine

receptor thought to be involved in hematopoieses is frequently overexpressed in SCLC^{74,82}.

Table 2. Oncogene mutations or overexpression in lung cancer

Oncogenes	Mutation	Abnormal expression frequency	
		NSCLC (%)	SCLC (%)
K-ras	point mutation (codon 12)	30-50	—
Myc	amplification	10	10-40
c-erb-2	increased expression	25	—
bcl-2	protein expression	25	—
Prad	overexpression/amplification	1 expression	—
Kit	overexpression	rare	frequent

Sources:^{48,72-77}

Of the various genetic alterations occurring in NSCLC, mutations in the K-ras oncogene and the p53 tumor suppressor gene are the most common genetic changes. Mutations in the p53 gene occur in 50-70% of all lung carcinomas, 50% of which are point mutations. These mutations are dispersed throughout exons 5-8 of the p53 gene. Cells with a point mutation have the advantage that they can be detected in a large population of normal cells in contrast to e.g. deletions. Mutations of the K-ras oncogene are discussed in the following section.

2.1.1 K-ras point mutation detection in lung carcinomas

Point mutations in the K-ras gene are among the most common genetic alterations associated with the development and progression of human cancer^{88,89}. The K-ras gene (homologous to Kirsten murine sarcoma virus oncogene) is a member of the ras family consisting of 2 other functional ras genes including H-ras (homologous to Harvey murine sarcoma virus oncogene) and N-ras (originally isolated from a neuroblastoma cell line). The ras genes code for highly related proteins with a relative molecular weight of 21 000 (p21). These proteins undergo a number of posttranslational modifications at the C-terminus, including farnesylation which result in their localization to the plasma membrane⁹⁰. The p21 coding sequences of each of these genes are equally distributed in 4 exons except K-ras-2 gene, which possesses 2 alternative fourth coding exons (exons IVA and IVB) that allow synthesis of

2 isomorphous p21 proteins of 188 and 189 residues that differ in their carboxy terminal domains.

The ras proteins H-, K-, and N-ras are central regulators in the cellular signal transduction process leading to cell growth and differentiation. They are molecular switches that in the resting cell are in the guanosine diphosphate (GDP)-bound inactive state. In response to receptor protein tyrosine kinases or other growth factor-dependent stimuli, these ras proteins become activated by binding guanosine triphosphate (GTP). In the GTP-bound active state, they interact with and activate downstream targets. In brief, activation of the ras proteins causes a cascade of activation of several proteins, which will ultimately lead to the phosphorylation of transcription factors. These factors in turn will enhance the transcription of several genes that play a role in cell growth. Ultimately, GTP hydrolysis mediated by ras completes the GTPase cycle.

The conformational state of ras is regulated by two kinds of proteins, guanine nucleotide exchange factors and GTPase-activating proteins (GAPs)⁹⁰. The rate at which guanine nucleotides bind to and dissociate from purified ras proteins is normally very slow (the half life for occupancy of the nucleotide binding site would be expected to be 1 hour under the ionic conditions found within a mammalian cell)⁹⁰. Guanine nucleotide exchange factors such as Sos or Cdc25 are positive regulators that increase the dissociation rate of guanine nucleotides, allowing GTP, which is more prevalent in the cell, to bind ras. GAPs are negative regulators that stimulate hydrolysis of bound GTP to GDP by accelerating the slow intrinsic rate of GTP hydrolysis by several orders of magnitude. Several mammalian GAPs for ras have been described such as p120^{GAP} and neurofibromin, the protein product of the type I neurofibromatosis (NF1) gene.

Point mutations occurring in several positions activate the ras oncogenes. They have impaired intrinsic GTPase and are insensitive to GAPs, and thus are unable to switch off the transmitted signal. Because of the action of the negative regulator GAP, wild type ras is found in the inactive GDP-bound state in unstimulated cells, whereas oncogenic ras remains longer GTP-bound. Thus, mutations confer transforming properties of the ras genes by yielding proteins with constitutive activation that generate continuous signals to proliferate. Those mutations occur in codon 12, 13, 59, 61 and 63, all of which lie near the interaction sites of ras proteins with the and phosphate residues of the bound GTP. Alternatively, some transforming mutations increase the dissociation rate of guanine nucleotides, which affect codon 116, 117, 119 and 146, all of which are sites involved in the interaction p21 ras with the purine base of GTP. Again, this leads to an accumulation of the active GTP-bound form of p21 ras.

Oncogenic mutants of ras are found in up to 30% of human tumors⁹¹, with the most common point mutations being at codon 12 and 13. Mutational

activation of ras occurs in carcinomas of the colorectum (40-50%), pancreas (90%) and lung (50%).

In lung cancer, K-ras mutations occur predominantly in codon 12^{80,84,92-95}. The frequency of K-ras mutations varies between different histologic subtypes, being activated in 20-57% of adenocarcinoma and large cell carcinoma^{80,81,93,96-100}. In addition, an association of the presence of K-ras alterations in adenocarcinoma was demonstrated with particular reference to cytologic subtypes^{98,101,102}. K-ras point mutation analysis in bronchioalveolar lung carcinomas showed that K-ras mutations are constant events in mucinous subtypes¹⁰³. The squamous cell carcinomas of the lung were initially reported to contain mutated K-ras only exceptionally^{17,96}, but more recently, they have also been found, at a lower frequency, in other forms of pulmonary neoplasia, including squamous cell carcinoma^{95,104}.

The time-point 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^{92,105-108}, i.e. just before the occurrence of visible tumors.

Using tissue specimens removed at biopsy or resection K-ras mutations appear relatively late in the multistep process of carcinogenesis and may be associated with the appearance of the malignant phenotype¹⁰⁹. This was also reported by others^{108,109} and is further confirmed based on the observation that mutations are rarely detected in preneoplastic epithelium adjacent to invasive lung cancer.

The occurrence of K-ras point mutations has also been described to be a very early event in the process of tumor formation, before the bulk of clonal expansion, and in very small precursor lesions⁹². Persistence of K-ras mutations in the majority of tumors and their metastasis suggests that these mutations are essential to maintain the malignant phenotype^{92,110}. Presence of K-ras in adenocarcinoma from former smokers abstinent for 15 years and longer suggests that these mutations occur early in the multistep sequence of events, i.e. before clonal expansion. Persistence of mutations at codon 12 of K-ras show that these changes are irreversible^{79,80,111}.

It has been reported recently that for K-ras alterations in NSCLC, G:C to T:A transversions are the most common changes^{84,97,112-114}. Based on results of K-ras activation in lung adenocarcinoma from former smokers, Westra et al reported the relationship between K-ras alterations and smoking, in which G-T transversions were identified¹¹¹. There is about a 5-fold increase in the K-ras mutation frequency among smokers relative to nonsmokers, which indicates association between tobacco use and incidence of K-ras mutations in adenocarcinoma^{13,95}. Beside the G-T transversions observed in lung carcinomas, other alterations have been found induced by other carcinogens in tobacco smoke¹¹⁵.

The prognostic significance of the presence and specific genotype of K-ras mutations in lung cancer has been the subject of intense study in recent

years^{22,78,80,81,95,97,112,113,116-125}. While no uniformity exist in the interpretation of these results, patients whose tumors are shown to harbour K-ras mutations have more aggressive disease with shorter disease-free survival and lower overall survival^{184,93}. Thus, detection of K-ras mutations in selected cancers may be a useful predictive marker of tumor behavior. In lung cancer, the type of K-ras appears to have a prognostic significance^{80,95,97}.

2.2 Genetic alterations in premalignant lesions

The concept of carcinogenesis implies that a stage exists in which precursor cells have begun to accumulate changes that may ultimately lead to invasive carcinoma, but these cells have not yet acquired the capacity to invade normal tissue or to metastasize. The aggregate of changes that occur in precursor cell populations prior to the development of invasive cancer are termed premalignant changes. These abnormalities have been initially studied in preneoplastic epithelium and later, in morphologic 'normal' epithelium. Many of the changes in preneoplastic epithelium have been summarized in table 3. Figure 1 illustrates the various stages in range of severity of the key lesions of preneoplasia, ranging from normal bronchial epithelium to hyperplasia, metaplasia, dysplasia/carcinoma in situ and invasive cancer.

Figure 1. Morphologic drawing of the development of invasive carcinoma of the lung. 1. Normal bronchial epithelium. 2. Hyperplasia, defined as $>12\%$ parabasal cells in airways larger than 4 mm, or $>6\%$ parabasal cells in airways smaller than 4 mm¹²⁶. 3. Metaplasia, the replacement of the usual respiratory epithelium by non-keratinized squamous epithelium. 4. Dysplasia, disorganized squamous epithelium usually with cytonuclear atypia. Carcinoma in situ (CIS), identified when cells with all the nuclear and cytoplasmic features of malignancy are present but limited to a given surface by an intact basement membrane. 5. Invasive cancer, growth beneath basement membrane.

Table 3. Genetic changes in premalignant human bronchial epithelial cells

Target	Genetic alteration	Reference
3p	LOH	52,127,128
5q	LOH	129
9p	LOH	63
13q	LOH	129
17p	LOH	129,130
p53	mutation	130,131
p53	↓ reactivity	132-135
microsatellite alterations	present	136,137
telomerase	↓ expression	138
p21 ^{Waf1/Cip1}	↓ expression	139
hnRNP A2	↓ expression	140-142
matrix proteases	↓ expression	143
DNA content	aneuploidy	144
cytosine	↓ methylation	145

LOH: loss of heterozygosity, points to a deletion of (part of) a chromosome or one allele of a gene

2.3 Time-point of genetic alterations

Initially, genetic alterations were demonstrated in morphologically altered epithelial lesions. Kishimoto and coworkers reported hyperplasia as the earliest epithelial lesion showing 3p LOH in four of seven cases (57%), and remaining cases showing more advanced morphological change¹²⁸. Hyperplasia in the respiratory epithelium was the earliest lesion in only two of seven cases (28%) with 9p LOH⁶³. Other molecular and cytogenetic studies have demonstrated p53 mutations and 3p deletions in dysplastic lesions^{127,130,131,146}. Since different genotypic alterations were found in preneoplastic lesions and tumor specimens from the same patients, the biologic significance of these alterations is not clear yet. Possibly, these alterations could be a marker for genetic damage, indicating a high risk. More recently, presumed early genetic alterations have been described in microscopically normal bronchial epithelium of heavy smokers with loss of heterozygosity on chromosome 3p, 5q, 9p and 17p^{57,129}. Interestingly, the occurrence of 3p deletions in microscopically normal

bronchial epithelium was present in >80% of the smokers while only 10% will ultimately develop lung cancer.

The use of the term 'early' and 'late' in the literature on carcinogenesis/tumorigenesis depends on the scope of the researchers. Mutations in the K-ras gene seem to be a late event compared with the above mentioned far more early chromosomal deletions demonstrated in heavy smokers^{57,129,147}. In addition, ras mutations are not frequently found in adjacent dysplasias¹⁰⁸, but have been shown in atypical alveolar hyperplasia^{148,149}. With respect to tumorigenesis the K-ras mutation can be called an early event^{92,108}.

Data from immunohistochemical studies on p53 accumulation show a progressive increase in the frequency of staining from 0% of normal bronchial mucosa to 2/3 of severe dysplasias, carcinomas in situ and microinvasive carcinomas^{130,131,134,146}. Most of these studies support a multistage model for SqCC in which p53 mutation occurs in a minor part of the earliest neoplastic lesions and the hypothesis is that the large majority of mutations occur before invasion capacity develops, favoring p53 also to be a relatively late event in lung carcinogenesis, just before tumorigenesis⁹².

In previous studies, overexpression of a 31 kD tumor associated antigen, identified as heterogeneous nuclear ribonucleoprotein (hnRNP) A2 was identified prior to any morphologic abnormality in most lung cancer cases. With this antibody a rather low specificity of 88% and a sensitivity of 91% were reported^{141,150,151}.

In summary, from the acquired mutations K-ras and p53 are relatively late events in lung carcinogenesis and may be used for early detection of lung cancer. In contrast, deletion of presumed tumor suppressor genes on chromosomes 3p and 9p are very early events with lower specificity for lung cancer than the more late events.

2.3.1 Field cancerization and chemoprevention

The term field cancerization was originally used to describe the occurrence of multiple carcinomas arising in patients with oral squamous cell carcinoma¹⁵². These carcinomas can arise synchronously or metachronously in people with head and neck or lung cancer. According to the field cancerization theory the entire upper aerodigestive tract epithelium has been mutagenized, thereby placing the affected individual at risk for the development of one or more cancers.

The term chemoprevention has been used since almost two decades¹⁵³ and has together with the advances in molecular biology and their application to cancer, become more and more popular in recent years. Chemoprevention is defined as the prevention of cancer in individuals at risk, through the administration of compounds which inhibit the process of carcinogenesis. When the most common early mutations in lung carcinogenesis are defined, it will be possible to determine with what frequency epithelial clones with

mutation expand. In addition, identification of premalignant molecular changes that correlate strongly with the development of cancer would therefore allow both better monitoring of chemoprevention studies and more precise selection and stratification of the population at risk. This is an area of research in development.

3. Lung cancer screening

3.1 Primary prevention

The most important intervention to halt lung cancer is smoking cessation. Due to aggressive governmental efforts of education and antismoking programs, and the recognition that passive smoking is dangerous, there has been a reduction in overall smoking, particularly in men. However with all the industrial, social and economical impact and the addictive effect of nicotine, the overall smoking habits are apparently difficult to diminish with medical and scientific information. In addition, the risk of lung cancer remains elevated several years after smoking cessation (latency period)¹⁵⁴. Furthermore, the recently reported increase in smoking rates among teenagers emphasize the continued importance of lung cancer¹⁵⁵. Thus, many people will still develop lung cancer due to smoking. In addition, there are other causes of lung cancer, like exposure to asbestos, uranium and chloroethyl ether which are mostly found in big cities than rural places^{9,156-161}. For all these reasons secondary prevention by means of 'screening' is necessary.

3.2 Results of screening studies for lung cancer

Screening seems a logical approach to decrease the mortality from lung cancer based on the assumption that survival for individuals in whom lung cancer is diagnosed at an early, asymptomatic stage is better than for those in whom the diagnosis is made at the time of clinical signs and symptoms.

One of the first major screening trials for lung cancer was performed in England during 1959-1963¹⁶². To assess whether six monthly routine chest radiography could improve the present poor prognosis of bronchial carcinoma, this service was offered to men aged older than 45 years in X-ray units in South London. Among 50,550 men that were examined at least twice, 147 patients with primary bronchial carcinoma were detected, 27% of whom lived four years or longer. The overall four year survival rate of the whole group was 18%, compared with 9% for all patients in the region. Since there was no adjustment for self-selection bias, lead time bias (lead time is the duration between detection through screening and clinical detection), overdiagnosis bias (the risk of identifying lesions on screening that never become clinically

evident), or length bias (only applies to the prevalence cases picked up on the first screening when the more slowly growing cancers are overrepresented), a significant impact of screening could not be demonstrated by this study. Follow-up period was relatively short (4 years), so a long term effect was not likely to be revealed¹⁶³.

In the 1970s, the National Cancer Institute sponsored three major screening studies, known as the Cooperative Early Lung Cancer Group, which aimed to evaluate screening for lung cancer with chest roentgenographs and sputum cytologic examinations¹⁶⁴. These studies were carried out at the Mayo Clinic, Johns Hopkins Hospital, and Memorial Sloan-Kettering Cancer Center^{164,165}. Enrollment in the studies was confined to men, 45 years of age or older, who were smoking at least one package of cigarettes daily (or who had smoked this much during the preceding year). The randomized trials at Hopkins and Memorial evaluated the benefit of adding sputum cytologic evaluations every fourth months to early chest radiographs. The trial at the Mayo Clinic evaluated the effect of cytologic and radiographic screening every fourth months compared with a recommendation of yearly chest radiograph and sputum cytology. None of these trials evaluated screening in comparison with a no-screening control arm. Radiographic screening detected significantly more lung cancers than did cytology alone, and the two tests together had the greatest sensitivity. Neither the addition of cytology to chest radiograph nor intensive screening with more modalities improved lung cancer mortality. With chest roentgenography peripheral NSCLC were detected, while sputum cytology predominantly revealed centrally located, intrabronchial squamous cell carcinomas. Those three clinical trials demonstrated among the 30,000 high-risk participants that, although screening of chest radiography and sputum cell morphology can detect presymptomatic, earlier-stage carcinoma, higher resectability and survival rates among the study groups than in the controls did not translate into lowered (overall) lung cancer mortality¹⁶⁶. Length-biased sampling, lead-time bias, and misclassification, when added to failures of detection and of therapy, resulted in a lack of significant improvement in mortality rates^{164,165,167,168}. The 5-year survival rate improved slightly in those who participated in lung cancer screening, the overall long term mortality of lung cancer was essentially unchanged in this study. Accordingly, it was argued that chest roentgenographic and sputum cytology screening for lung cancer was not helpful and therefore should not be recommended.

A more recent prospective randomized study conducted in Czechoslovakia compared a policy of screening to no intervention in the absence of symptoms¹⁶⁹. All enrolled men underwent initial prevalence examination, after which the control group had no other planned intervention for three years. Screened individuals underwent chest radiography and cytology every six months. At three years, chest radiography and sputum cytology were performed in both groups, followed by annual chest

radiography. At six year follow up, this study also failed to demonstrate a difference in lung cancer mortality in the population screened by both cytology and chest radiography or the control group with no screening¹⁶⁹.

Another approach to lung cancer screening (based on symptoms, spirometric abnormalities) was conducted in Grand Junction, Colorado, using sputum cytology as a case-finding tool in the identification of roentgenographically occult lung cancer¹⁷⁰. In brief, 51 patients with standard chest film occult lung cancer were identified as having occult lung cancer by sputum cytology. Cancer was diagnosed by one or more standard light bronchoscopy and biopsy or brushing, using a flexible fiberoptic bronchoscope. The majority of cancer cases detected were squamous cell carcinoma because of its central location and therefore a higher likelihood of easy exfoliation of tumor cells into sputum. Adenocarcinoma is less likely to be diagnosed by sputum cytology because it is usually peripherally located. Two large cell carcinomas and two undifferentiated carcinomas completed the series. From this group of patients, a total of 86% had early stage disease and thus were amenable to surgical therapy. Apart from the surgically treated group, other patients received attempts at curative radiation therapy for various reasons. An actuarial survival of both groups, including deaths from all causes was 55%¹⁷⁰. These results suggested that the overall 5-year survival rate for all patients would be significantly higher than the 10% to 15% survival rate at 5 years that is well known to be the outcome when lung cancer is diagnosed by conventional methods¹⁷¹. However, the final outcome of all 51 patients awaits further follow-up¹⁷⁰.

Monoclonal antibodies directed against oncofetal antigens (i.e. a difucosylated Lewis X and a 31-Kd glycoprotein antigen) expressed on lung cancer cells, which have been earlier described, were also used in screening studies to enhance the specificity and sensitivity of sputum cytology^{151,172}. Although this technique significantly improved the sensitivity of regular screening by sputum cytology, still a rather low specificity of 88% and a sensitivity of 91% were reported^{150,151}. The clinical value of sputum immunocytology was also investigated in a group of resected stage I lung cancer patients, who have a very high risk to develop secondary pulmonary neoplasms¹⁴¹. The results of this study showed that expression of those antigens was not specifically related to neoplastic epithelium, but also occurred in nonneoplastic epithelium of the respiratory tract¹⁴¹.

The screening trials based on X-ray of the thorax combined with cytologic investigation of sputum can detect presymptomatic, earlier-stage carcinoma leading to higher resectability and survival rates among the study groups than in the controls. However, this did not result in reduction of mortality. To be seen on a routine chest radiograph, in the favorable circumstance of a peripheral nodule that is not in the presence of shadows of rib or mediastinal structures, a lesion has to be approximately 1 cm in diameter. Such a mass will typically contain 10^9 tumor cells. For this reason these signs are called

'late signs' in the early detection. Thus lung cancer must be detected in an earlier preclinical phase than with conventional X-ray technique, when the tumor is smaller and not yet metastasized. The long preclinical history for even the smallest radiographically detectable tumors results in a considerable opportunity for the accumulation of mutations and clonal selection of phenotypes capable of invasion, metastasis, and drug resistance.

3.3 Genetic alterations as biomarkers in lung cancer

Since the screening programs using roentgenography and sputum cytology have not been successful, other strategies for reducing the high numbers of lung cancer deaths are being developed. These include beside continuous primary prevention programs the identification of high risk populations. Primary prevention is the most desirable solution for lung cancer, but despite the efforts of education and anti-smoking campaigns it is not likely that the exposure to tobacco smoke will disappear during the next century. Therefore, secondary prevention is the only approach which ultimately may be effective for early detection of lung cancer.

Nowadays, genetic alterations are used as biomarkers for identification of populations with an increased risk for lung cancer. One of the genetic alterations used as a possible biomarker for lung cancer is point mutations of the K-ras gene, as mentioned in section 2.1.1. Previously, several methods such as allele-specific oligonucleotide hybridization or direct sequencing of amplification products have been utilized for the detection of K-ras point mutations in lung cancer^{13,17,80,81,84,97,111-113,173}. However, among several different procedures for detection of these alterations, difficulties in critical analytic steps following DNA amplification have prevented the technology from being more widely applied in clinical laboratories and the biotechnology industry. In general, analysis of DNA products following amplification usually takes one of three forms (1) colorimetric detection, (2) direct sequencing and (3) electrophoresis gels. Each of these procedures has proved more difficult than initially anticipated, in part because of special properties of amplified DNA that differ from nonamplified DNA. Several well-known difficulties in analyzing amplified DNA have been documented, resulting in the following:

1. Base pairing at extreme DNA ends is believed to be unstable during amplification reaction.
2. Incompletely used substrates and reaction byproducts of amplification procedures can interfere with subsequent analysis. Interfering reactants include nucleotides and oligonucleotides; interfering byproducts include pyrophosphates.
3. Extraneous DNA, including 'primer dimer', is often coamplified with target, confusing subsequent analysis and competing with target in

sequencing steps and point mutation analysis. For this reason, amplified DNA must be extensively purified prior to these procedures.

4. Amplified DNA strands tend to rapidly reanneal and exclude hybridized probes. This effect has been noted to create special difficulties in the sequencing of double-stranded amplification products. Numerous strategies have been suggested for convening amplification fragments into single strands so that they can be more easily analyzed to hybridization. However, none has proved entirely satisfactory, resulting in a relatively low sample sensitivity of these methods, i.e. the ability to discriminate cells containing a mutation in a background of genetically normal cells, which could lead to underestimation of positive cases.

For detection of K-ras mutations in a heterogeneous cell population, where only a small fraction of tumor cells containing the mutation are present among much larger numbers of genetically normal cells, a highly sensitive technique is required, which should be very specific but not time-consuming, technically simple and safe.

4. Aims of the present study

The aims of the present study are 1) to develop a new, rapid, reliable method for detection of known point mutations which may be clinically useful, 2) to determine the sensitivity of this point mutation detection method, 3) to validate this method for K-ras point mutation detection in NSCLC, and 4) to examine whether this method can be used for K-ras point mutation analysis in sputum of patients with adenocarcinoma of the lung. The method used for point mutation detection in this thesis is named Point-EXACCT. The development of Point-EXACCT using the K-ras gene as a model system is described in chapter 2. To use this method for early detection, a highly sensitive assay is required. To this end, the sensitivity of this detection method is examined by reconstruction experiments and described in chapter 3. For further use of this model system on patient material, the method should be compared with already available techniques which are called golden standard techniques for point mutation detection. The validation of the Point-EXACCT method for K-ras point mutation detection in NSCLC is further described in chapter 4. In chapter 5, it is studied whether K-ras point mutations can be found with the Point-EXACCT method in sputum of patients with adenocarcinoma of the lung. In chapter 6, we investigate the presence/absence of K-ras point mutations in normal appearing epithelial cells from bronchial brushes of patients with NSCLC. In chapter 7, the additional diagnostic value of K-ras point mutations in bronchial wash fluids of patients under suspicion of lung carcinoma was examined. Chapter 8 correlates the presence of K-ras point mutations found in NSCLC patients with prognosis.

Two

Development of Point-EXACCT

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A rapid, reliable method for detection of known point mutations : Point-EXACCT

*Veerle AMC Somers, Peter TM Moerkerk, James J Jr Murtagh
and Frederik BJM Thunnissen*

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Point mutations in the human genome play a central role in tumorigenesis¹⁷⁴. Several methods are available for detection of known point mutations¹⁷⁵⁻¹⁷⁷, which are to a variable extent time-consuming, technically complex, or hazardous due to the use of radioactive materials. To circumvent these problems we describe a simple, highly specific non-radioactive microtiter plate format for detection of PCR products that offers a high sensitivity towards the detection of known point mutations and illustrate this technique for the detection of human K-ras oncogene. The detection format is based on an extension of the EXACCT procedure¹⁷⁸. In short: after exonuclease digestion, polymerase chain reaction fragments are determined by hybridization with a capture and a detection probe complementary to sequences near the 3' end of the antisense fragment. The capture probe bears a biotin residue and the other probe digoxigenin. After hybridization the PCR product hybrids are captured in streptavidin-coated microtiter plates and detected with labeled anti-digoxigenin antibody. For the detection of known point mutations this procedure was extended by using after the capture step the ligation of a mutation specific capture probe with adjacent detection probe (Point-EXACCT, see figure 1)¹⁷⁹. As a model system base 1 of codon 12 of the c-Ki-ras gene is used.

Figure 1. In (a) the EXACCT procedure is explained with two options : format A and format B. DNA from the cell lines was amplified using primers of the first exon of c-Ki-ras gene outside the codon 12 region. Following amplification, the reaction products were digested to single strands with T7 gene 6 exonuclease. Separate hybridization was performed with a common digoxigenin-labeled detection probe and one of four different biotinylated probes containing the wild type or point mutation. After transfer to a coated microtiter plate (coupling) and washing, the products were incubated with T4 DNA ligase. In format B the exonuclease digestion can be limited to one strand by adding phosphorothioate linkages (SSS) to one primer. (b) shows that in case of perfect complementarity in Point-EXACCT, the enzyme T4 DNA ligase covalently joins the 5' biotinylated probe and the 3' detection probe. If the probes and target are mismatched at their junction, a covalent bond is not formed. After ligation, denaturation and subsequent washing, amplification products are detected by POD-conjugated anti-digoxigenin antibody.

WT = wild type; Mut = mutation

Purified DNA from 3 different cell lines with known point mutations was used to test Point-EXACCT. HL60 has the wild type DNA for codon 12 of the first exon of the c-Ki-ras gene (GGT)⁸⁸. A549 contains a homozygous serine mutation (AGT)¹⁸⁰ and Calu-1 contains a cysteine mutation (TGT). Calu-1 is heterozygous at the c-Ki-ras locus, harboring both a wild type and activated allele¹⁸¹. After PCR with sense primer (5' TTT TTA TTA TAA GGC CTG CTG 3') and antisense primer (5' TCA GAG AAA CCT TTA TCT GTA TCA AAG AAT GG 3') the PCR product was digested on both sides with exonuclease, leading to single strands. As an alternative, exonuclease digestion can be blocked on one strand by enzyme phosphorothioate linkages into one PCR primer. Previously, 3'-5' linkages have been shown sufficient to inhibit exonuclease digestion¹⁷⁸. The digested product was then heated to stop the reaction and separately hybridized with one common digoxigenin labeled probe

(5' pG TGG CGT AGG CAA GAG TGC CTT G-DIG 3') and with one of 4 different biotinylated capture probes (5'-BIO- TA TAA ACT TGT GGT AGT TGG AGC TG-3'), containing the wild type (G) or point mutation (A, T, C) at the 3' side base that aligns with the site investigated for a point mutation. The hybridization efficiency after exonuclease digestion was far greater when compared to heat denatured amplification products.

Essential in the Point-EXACCT technique is that (i) only molecules hybridized with two probes and (ii) are subsequently ligated, will be detected by this format giving a very high degree of specificity. T4 DNA ligase catalyzes the formation of a phosphodiester linkage between the two adjacent oligonucleotides, the 5' biotinylated probe containing the point mutation at the 3' side and the 3' labeled detection probe: if there is perfect complementarity, the enzyme T4 DNA ligase covalently joins the 5' biotinylated probe and the 3' labeled detection probe. If the probes and target are mismatched at their junction, a covalent bond is not formed. Variation in ligase concentration, reaction temperature and time revealed that the best signal/noise ratio was obtained with incubation of $1,66 \times 10^{-1}$ U T4 DNA ligase for 15 min at room temperature. The use of a higher ligase concentration resulted in non-specific reactivity leading to a higher signal in controls, whereas ligation with lower enzyme concentration resulted in reduced sensitivity. Changes in temperature and reaction time did not improve the sensitivity or specificity. The best sensitivity (1:10,000 diluted amplification product) was obtained with 1% blocking reagent or 5% heat inactivated fetal calf serum during the antibody incubation, leading to a lower background.

The results of Point-EXACCT in the K-ras model system are shown in figure 2. Cell line HL60 showed only a signal after incubation with the wild type probe. The first mutation, GGT to AGT, was only detected in amplified DNA of cell line A549. In Calu-1, both wild type GGT and the for cysteine coding sequence (GGT to TGT) were detected, referring to heterozygosity for this sequence. No cell line containing the GGT to CGT mutation was tested with the mutation specific probes. However, incubation of the mutation specific probe CGT gave no signal above the background values using DNA of either cell line HL60, A549 or Calu-1 (see figure 2). The whole procedure takes 10 hours.

Point-EXACCT requires considerably less time and effort than other techniques used for the detection of known point mutations. This method can be easily automated, permitting rapid screening of tissue banks with multiple probes to individual base substitutions, deletions or additions. The simplicity of Point-EXACCT makes it a highly promising method for the detection of known point mutations.

Figure 2. The relative optical density of the Point-EXACCT procedure obtained after ligation is shown for three different cell lines. A 204 bp region of the c-Ki-ras gene was amplified using oligonucleotides of the first exon of c-Ki-ras gene outside the codon 12 region. Purified genomic DNA (1 μ g) was added to 80 μ l of 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 250 pmole of each deoxyribonucleotide, 19.8 μ mol/l of antisense primer, 28.1 μ mol/l of sense primer and 2.5 U of Taq DNA polymerase (Gibco BRL, Life Technologies, Inc.). PCR was performed for 40 cycles (94°C, 30 sec; 55°C, 30 sec; 72°C, 40 sec) in an automated DNA Thermal Cycler (Perkin-Elmer Cetus). After amplification, the products were supplemented with dithiothreitol to a final concentration of 1 mmol/l and digested with 4U/ μ l T7 gene 6 exonuclease (United States Biochemical, Cleveland, Ohio) for 15 minutes at 37°C in order to obtain single strands. After heat denaturation at 75°C for 15 minutes 10 μ l of digested amplification product was added to 38 μ l hybridization buffer (4x SSC, 20 mM HEPES, 2 mM EDTA, 0.15% Tween 20), 1 μ l (50 ng/ μ l) digoxigenin-labeled detection probe and 1 μ l (50 ng/ μ l) of a biotinylated probe. Separately incubation was performed with 4 different biotinylated probes, containing the wild type (G) or point mutation (A, T, C) sequence. The products were transferred to a 96-well flat-bottomed microtiter plate (Falcon) coated with 80 μ l biotinylated bovine serum albumin (BSA 2 μ g/ml, Sigma Chemical Co.), in phosphate buffered saline (PBS) for 1 hour at 37°C, washed 3 times with 100 μ l of PBS containing 0.05% Tween 20, and saturated with 50 μ l streptavidin (1 μ g/ml) with 0.5% gelatin/well for 1 hour. Amplification products were captured in the wells. After washing the plate 3 times with 100 μ l PBS/Tween 0.05%, the products were ligated for 15 minutes with 1.66×10^2 Units T4 DNA ligase (Promega Corporation, Madison, USA) in 50 μ l 1x ligase buffer (30 mM Tris HCl, pH 7.8; 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM ATP). A thermostable Taq DNA ligase (New England Biolabs) was also tested. The wells were then washed with 100 μ l PBS/0.05% Tween 20 and denatured with 50 μ l 0.07 M NaOH for 2 minutes, washed

twice with 100 μ l 0.01 M NaOH/0.05% Tween 20 and once with PBS/Tween 0.05%. The products were incubated with 80 μ l/well of a 1 : 2000 dilution of peroxidase (POD)-conjugated anti-digoxigenin antibody Fab fragments (Boehringer Mannheim Biochemicals, Indianapolis, USA) in 100 mM Tris HCl, pH 7.5/150 mM NaCl supplemented with 1% blocking reagent (Boehringer Mannheim Biochemicals, Indianapolis, USA) for 1 hour and washed 3 times with 100 μ l PBS/Tween 0.05%. After washing, 100 μ l/well of a 3,3',5,5'-tetramethyl-benzidine dihydrochloride (TMB) chromogen solution (10 mg/ml) was added and the color development stopped with 50 μ l/well 2 M phosphoric acid. Previous steps in microtiter plate were performed at room temperature with shaking. The plates were read at 450 nm in a BIO-RAD Novapath Microplate Reader. Optical density from the ligated products was expressed relative to the (maximal) optical density of the controls: a hybridized sample without ligation (i.e. EXACCT only). In each experiment, PBS was used as negative control. In addition, the biotin probes mismatching to the cell line tested can be regarded as control as well.

Three

Sensitivity of Point-EXACCT

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Exonuclease enhances hybridization efficiency :
Improved direct cycle sequencing and point mutation detection
*Veerle AMC Somers, Darcy A Leimbach, James J Jr Murtagh
and Frederik BJM Thunnissen*
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Abstract

Solution hybridization is an essential step in sequencing and some point mutation detection methods. In practice this hybridization is hampered resulting in the need of additional purification of the amplification products. The use of T7 gene 6 exonuclease may lead to efficient production of single-stranded DNA. In this study the effect of pretreatment with exonuclease on direct cycle sequencing and point mutation detection was analyzed.

Exonuclease-treated products were directly cycle sequenced without further purification. This resulted in highly efficient quality improvement for sequencing allowing detection of heterozygotes. Point mutation detection by Point-EXACCT (exonuclease-amplification coupled capture technique) demonstrated detection of one cell containing a mutation in an excess of 75,000 wild type cells.

Exonuclease-enhanced detection methods offer simple, rapid detection strategies that are easily adaptable for widespread clinical laboratory use. With the use of exonuclease, the detection of heterozygosity using fluorescent cycle sequencing is becoming more reliable. The high sensitivity of Point-EXACCT due to the use of exonuclease makes it a highly promising method for large-scale screening of (pre)malignant changes in patients with a high risk for developing cancer.

1. Introduction

Screening methods for genetic mutations such as point mutations, insertions, deletions or detection of polymorphisms are considered to play an increasingly important role in prevention and treatment of many hereditary genetic disorders and acquired malignancies. Several methods use PCR to amplify (part of) the gene of interest, with further analysis of the product. In this process solution hybridization of the amplified product is an essential step for both sequencing and point mutation detection methods.

DNA amplified by PCR consists of relatively short double-stranded fragments, being able to rapidly reassociate after heat denaturation, thereby reducing hybridization efficiency. Furthermore, other components of the amplification reaction including deoxynucleotides and primers could impede hybridization. Therefore, several strategies have been developed to obtain single-stranded amplified DNA as template for hybridization in contrast to traditional procedures using heat-denatured amplification products.

For sequencing, asymmetric PCR has been used to obtain single-stranded DNA following PCR¹⁸². The major weakness of asymmetric PCR is that only relatively small amounts of single-stranded DNA are produced and extensive

optimization of conditions is required for the individual DNA template to be sequenced. Another method aimed at converting PCR products into single strands via magnetic beads is commonly used. However, while this method gives reliable sequence data, it has proved to be expensive and tedious^{183,184}. Recently, a new method for direct cycle sequencing of PCR fragments using mutant Taq enzyme was described¹⁸⁵. This method allowed direct sequencing of PCR products, but optimization of the amplification reaction was crucial to obtain reliable detection of heterozygous sequence differences.

The 'oligonucleotide ligation assay' is a point mutation detection assay which is developed to circumvent the need for electrophoresis and critical hybridization conditions¹⁸⁶. Although this method is automated, the signal to noise ratio is still low. This could be explained by possible reassociation of the heat-denatured amplification products.

Thus, methods using solution hybridization can be improved if double-stranded amplification products are transferred to single-stranded DNA. As an alternative, T7 gene 6 exonuclease can be used to enhance sequencing and point mutation detection (Point-EXACCT)¹⁸⁷. T7 gene 6 exonuclease converts double-stranded amplified DNA to single strands to enhance specific hybridization. T7 gene 6 exonuclease digests either 5' phosphorylated or 5' hydroxylated DNA. This enzyme can be blocked by incorporating phosphorothioate (S)-linkages into one of the PCR primers. After amplification, the strand initially primed by the phosphorothioate primer in the PCR is resistant to exonuclease digestion, whereas the other strand is selectively digested away, resulting in the single-stranded amplification products.

The purpose of this study was to investigate the influence of exonuclease pretreatment of amplification products on hybridization conditions in direct cycle sequencing and point mutation detection. To this end, the sensitivity and efficiency of two different methods of fluorescent cycle sequencing with and without the use of T7 gene 6 exonuclease were compared, i.e. detection of heterozygous mutations using sequencing of double and single-stranded PCR, and the sensitivity of Point-EXACCT was examined.

2. Materials and Methods

2.1 Patients and tissues

From 10 patients consecutively treated with lobectomy or pneumectomy for NSCLC in the University of Maastricht tumor samples were obtained after resection, frozen and stored at -70°C. Twenty μm cryostat sections were prepared from each frozen tumor sample. Likewise, for histological examination after hematoxylin-eosin staining a 5 μm section was taken from each tissue. To avoid cross contamination the cryostat knife was cleaned with

alcohol 100% between sectioning of all different tumor samples. Genomic DNA was isolated by overnight incubation of the tissue sections at 56°C in 50-100 μ l proteinase K buffer containing 50 mM Tris HCl, pH 8.5, 1 mM EDTA and 0.5% Tween 20, depending on the size of the tissue sections. Samples were further denatured for 10 minutes at 95°C, and quickly stored on ice. Subsequently, products were centrifuged at 16,000 x g for 30 sec and again stored on ice. To estimate the concentration of DNA in the supernatant, 1 μ l was spotted on a 1% agarose plate with a serial dilution of a DNA-standard for comparison.

2.2 Cell lines

DNA from 5 different cell lines with wild type or mutated sequences for base 1 and 2 of codon 12 of the K-ras gene were used for PCR. DNA was isolated using standard phenol-chloroform extraction. HL60 and NCH-H716 cells have the wild type DNA for codon 12 of the K-ras gene (GGT)^{88,188}. A549 contains a homozygous serine mutation (AGT)¹⁸⁰ and Calu-1 contains a cysteine mutation (TGT). Calu-1, harboring both a wild type and activated allele was used, as demonstrated with sequencing¹⁸¹. SW480 is homozygous for the K-ras codon 12 valine mutation (GTT)¹⁸¹.

2.3 Reconstruction experiments

Cell lines A549, Calu-1 and SW480 were used for reconstruction experiments with the wild type cell line HL60. Cell line H716 showing the wild type for K-ras was used for the converse reconstruction experiment with cell line A549, having the homozygous mutation. After culturing and short trypsin digestion for Calu-1, A549 and SW480, cells were centrifuged, the pellet was resuspended in 1 x PBS and the concentration determined in a Burk counting chamber. Subsequently, all cell lines were diluted to a concentration of 2×10^5 cells in 1 ml 1 x PBS. The cell lines with a K-ras codon 12 mutation were diluted starting with 1 ml cells in 1 x PBS (2×10^5 cells) in 4 ml wild type cells in PBS. This 1:5 dilution was repeated several times resulting in a stepwise reduction of the number of cells containing a mutation. For the converse reconstruction of wild type cells (H716) with A549 the procedure was similar. The reconstruction experiments were repeated using new cell cultures with subsequent counting, mixing, DNA extraction, amplification and point mutation detection.

2.4 K-ras amplification

A 204 bp region of the K-ras gene was amplified as described previously with slight modification¹⁸⁷. Amplification was performed using oligonucleotides

of the first exon of K-ras flanking the codon 12 region: sense primer KPR3 (5' TTT TTA TTA TAA GGC CTG CTG 3') and antisense primer KI8 (5' TCA GAG AAA CCT TTA TCT GTA TCA AAG AAT GG 3'). PCR was performed using 40 cycles (94°C, 30 sec; 55°C, 30 sec; 72°C, 40 sec) in an automated DNA Thermal Cycler (Perkin-Elmer Cetus). Purified genomic DNA (500 ng-1.0 µg) was added to 80 µl of 10 mM Tris HCl (pH 8.3), 50 mM potassium chloride, 1.5 mM magnesium chloride, 250 pmole of each deoxyribonucleotide (dNTP), 1.1 µM of sense primer, 0.8 µM of antisense primer and 2.5 U of AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, USA). The amplification products were subjected to electrophoresis in a 2% agarose gel and visualized by staining with ethidium bromide.

For fluorescent cycle sequencing, 5 µl amplification product was used for cleanup with 1 µl exonuclease I (10 U/l), (United States Biochemical, Cleveland, OH) for 20 minutes at 37°C in order to remove excess oligonucleotide primers and dNTPs. After heat denaturation at 80°C for 20 minutes and cooling down to 4°C, double-stranded amplification products were available for fluorescent cycle sequencing.

2.5 K-ras amplification for single-stranded fluorescent cycle sequencing using exonuclease

A 233 bp region of the K-ras gene was amplified using oligonucleotides of the first exon of the K-ras gene outside the codon 12 region. Purified genomic DNA (200 ng) was added to 16 µl of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 250 pmole of each deoxyribonucleotide, 0.2 µM of sense primer ZKPR3 (5' CGA GGG AAG AGGT - TTT TTA TTA TAA GGC CTG CTG 3') containing a unique 12-base sequence (underlined) attached to the 5' site of the primer complementary to the target DNA, 0.2 µM of antisense primer KI8 and 0.5 U AmpliTaq DNA polymerase. A Perkin-Elmer GeneAmp 9600 was used for thermal cycling with an initial denaturation at 94°C for 2 min followed by 20 cycles denaturation at 94°C for 35 sec, primer annealing at 55°C for 35 sec and primer extension at 72°C for 45 sec. A final extension step was performed for 5 min at 72°C. Subsequently, 5 µl sample of the amplification product was taken for gel electrophoresis and 40 µl fresh amplification mix was added, containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 250 pmole of each deoxyribonucleotide, 1 µM sense adapter T-FOR (5' CAT CAT CAT CAT GCG AT CGA GGG AAG AGG 3'), containing phosphorothioate linkages (italic), which has the unique 12-base sequence at the 3' site (underlined), 1 µM antisense primer KI8 and 1.25 U AmpliTaq DNA polymerase. Amplification was performed for 20 cycles using the unique 12-bp sequence as a template for annealing with the adapter primer T-FOR. After amplification, 5 µl amplification product was used for cleanup with 1 µl exonuclease I (10 U/µl), (United States Biochemical, Cleveland, OH)

and 1 μ l of shrimp alkaline phosphatase (SAP-2U/ μ l), (United States Biochemical, Cleveland, OH) for 20 min at 37°C in order to remove excess oligonucleotide primers and dNTPs. After heat denaturation at 80°C for 15 min and cooling down to 4°C, 5 μ l cleaned product was digested with 10 U T7 gene 6 exonuclease (United States Biochemical, Cleveland, OH) for 15 min at 37°C in order to obtain single strands. Since the sense strand contains phosphorothioate linkages, this strand is protected against T7 gene 6 exonuclease, so after heat denaturation at 75°C for 15 min, only sense DNA strands were available for cycle sequencing (figure 1).

Figure 1. Exonuclease-enhanced DNA sequencing. Sulfur-bridges (*S) are incorporated in the sense strand by secondary PCR (a). Following amplification, the double-stranded product is treated with T7 gene 6 exonuclease. The phosphorothioated strand is protected from the action of this enzyme, whereas the opposite strand is hydrolyzed, resulting in single-stranded PCR products available for sequencing (b).

2.6 Fluorescent cycle sequencing

Cycle sequencing was performed for 25 cycles (96°C, 15 sec; 50°C, 1 sec; 60°C, 4 min) on a Perkin-Elmer 9600 thermal cycler utilizing the Taq Dye Deoxy terminator cycle sequencing kit (ABI) according to the manufacturer's directions. One μl of the diluted, EXO-SAP digested PCR-products was added to a MicroAmp reaction tube (Perkin-Elmer) containing 9.5 μl reaction pre-mix containing 5x TACS buffer (400 mM Tris-HCl, 100 mM ammonium sulfate, pH 9.0, 10 mM MgCl_2), 1 μl dNTP mix (750 μM dTTP, 150 μM dATP, 150 μM dCTP, 150 μM dTTP), 1 μl of each of the A, C, G and T taq Dyeoxy terminators, 1 μl (3.2 μM) of the sequencing primer K18 and 0.5 μl (2.5 U) of AmpliTaq DNA polymerase. Extended fragments were purified from unincorporated Dye Deoxy terminators and primer with the aid of Centri-Sep spin columns (Princeton Separations, Princeton, NJ) according to the manufacturer's directions. The reaction products were dried (SpeedVac) and resuspended in 5 μl loading buffer (formamide/50 mM EDTA, pH 8.0 (5:1, v/v)), heated to 95°C for 3 min, transferred to ice and loaded immediately on a 4% denaturing polyacrylamide gel. Gels were run for 12 h at 30 W constant power on an ABI 377 sequencer according to the manufacturer's instructions.

2.7 Point mutation detection using exonuclease

The procedure of Point-EXACCT is shown in figure 2 and is slightly modified¹⁸⁷. After amplification with primers of the first exon of the K-ras gene outside the codon 12 region, the products were supplemented with dithiothreitol (DTT) to a final concentration of 1 mmol/l and digested with 3U/ μl T7 gene 6 exonuclease (United States Biochemical, Cleveland, Ohio) for 15 minutes at 37°C. The digested product was then heated to 75°C for 15 minutes to stop the reaction. Subsequently, 10 μl of digested amplification product was added to 38 μl hybridization buffer (4x SSC, 20 mM Hepes, 2 mM EDTA, 0.15% Tween 20), 1 μl (6.5 fmol) digoxigenin-labeled detection probe and 1 μl (6.5 fmol) of one of the biotinylated probes, containing the point mutation at the 3' side (see table I). This was also done with probes directed against base 2 of codon 12 of the K-ras gene. Thus for base 1 and 2 of codon 12 of the K-ras gene, four different biotinylated probes were separately incubated, containing either the wild type (G) or one of the point mutations (A,T or C). The products were transferred to a 96-well flat-

bottomed microplate (Falcon) coated with 80 μ l biotinylated bovin serum albumin (BSA), (2 μ g/ml, Sigma Chemical Co.) in PBS for 1 hour at 37°C, washed 3 times with 100 μ l of PBS containing 0.05% Tween 20, and saturated with 50 μ l streptavidin (1 μ g/ml) with 0.5 % gelatin/well for 1 hour. Amplification products were captured in the wells and after washing the plate 3 times with 100 μ l PBS/0.05% Tween 20, the products were ligated for 15 minutes with 1.25 mU T4 DNA ligase (Promega Corporation, Madison, USA) in 50 μ l 1 x ligase buffer (30 mM Tris-HCl, pH 7.8; 10 mM MgCl₂, 10 mM DTT, 0.5 mM ATP) supplemented with 20% formamide. A thermostable Taq DNA ligase (New England Biolabs, Beverly, USA) was also tested. The wells were then washed with 100 μ l PBS/0.05% Tween 20 and denatured with 50 μ l 0.07M NaOH for 2 minutes, washed twice with 100 μ l 0.01 M NaOH/0.05% Tween 20 and once with PBS/0.05% Tween 20. The products were incubated with 80 μ l/well of a 1 : 2,000 dilution of peroxidase (POD)-conjugated anti-digoxigenin (DIG)-antibody Fab fragments (Boehringer Mannheim Biochemicals, Indianapolis, USA) in 100 mM Tris-HCl, pH 7.5/150 mM NaCl supplemented with 1% blocking reagent (Boehringer Mannheim Biochemicals, Indianapolis, USA) for 1 hour and washed 3 times with 100 μ l PBS/0.05% Tween 20. After washing, 100 μ l/well of a 3,3',5,5'-tetramethylbenzidine (TMB) chromogen solution (10 mg/ml) was added and the color development stopped with 50 μ l/well 2 M phosphoric acid. Previous steps in microplate were performed at room temperature with shaking. The plates were read at 450 nm in a BIO-RAD Novapath Microplate Reader (Bio-Rad Laboratories, California, USA).

Point-EXACCT

Calu-1

PCR

Exonuclease

Hybridization

Couple

After ligation, denaturation and capturing

Figure 2. Schematic representation of the Point-EXACCT method. The method is shown for cell line Calu-1, heterozygous for K-ras codon 12 base 1, containing both wild type guanine (G) and mutation thymidine (T). DNA from the cell line was amplified using primers of the first exon of K-ras gene outside the codon 12 region under conditions previously described. Following amplification, the reaction products were digested with T7 gene 6 exonuclease. Subsequently, an aliquot of digested product was added to hybridization buffer, a digoxigenin-labeled probe and a biotinylated probe containing the point mutation at the 3' side. Four different biotinylated probes containing the wild type or point mutation sequence were separately used for hybridization. Following hybridization, the products were transferred to a 96-well streptavidin-coated flat-bottomed microplate (coupling). After coupling and washing, the products were incubated with T4 DNA ligase. In case of perfect complementarity, the enzyme T4 DNA ligase covalently joins the 5' biotinylated probe and the 3'

detection probe. If the probes and target are mismatched at their junction, a covalent bond is not formed. After ligation, denaturation (to remove the non-ligated digoxigenin labeled probes) and subsequent washing, remaining products are detected by POD-conjugated anti-DIG-antibody.

Table 1. Sequences of the probes for the detection of point mutations in codon 12 of the K-ras gene with Point-EXACCT

K-ras codon 12 biotin-labeled mutation specific capture probes ^a		
Probe	Codon 12	Sequence
Base 1		
K12G	GGT	5'Bio-TA TAA ACT TGT GGT AGT TGG AGC <u>TG</u> -3'
K12A	AGT	5'Bio-TA TAA ACT TGT GGT AGT TGG AGC <u>TA</u> -3'
K12T	TGT	5'Bio-TA TAA ACT TGT GGT AGT TGG AGC <u>TT</u> -3'
K12C	CGT	5'Bio-TA TAA ACT TGT GGT AGT TGG AGC <u>TC</u> -3'
Base 2		
K12G	GGT	5'Bio-TA TAA ACT TGT GGT AGT TGG AGC <u>TGG</u> -3'
K12A	GAT	5'Bio-TA TAA ACT TGT GGT AGT TGG AGC <u>TGA</u> -3'
K12T	GTT	5'Bio-TA TAA ACT TGT GGT AGT TGG AGC <u>TGT</u> -3'
K12C	GCT	5'Bio-TA TAA ACT TGT GGT AGT TGG AGC <u>TGC</u> -3'
K-ras 3' digoxigenin-labeled detection probes		
Probe	Sequence	
K-ras Dig1	5' - pG TGG CGT AGG CAA GAG TGC CTT G-Dig 3'	
K-ras Dig2	5' - p TGG CGT AGG CAA GAG TGC CTT G-Dig 3'	

^a the specific mutation is underlined

3. Results and discussion

This report describes an important improvement of solution hybridization after exonuclease pretreatment of amplification products for fluorescent cycle sequencing as well as point mutation detection. Digestion of a double-stranded

amplification product to single strands by T7 gene 6 exonuclease increases hybridization efficiency and confers increased sensitivity and specificity of detection.

In general, the optimal template for polymerases used in sequencing of amplification products is single-stranded DNA. Therefore, we think the use of single-stranded amplification products as template for cycle sequencing is inevitable, especially in particular cases.

Figure 3 shows examples of the results after the reverse sequencing of the 233-bp fragment of the K-ras gene from cell line Calu-1 using (a) the Taq Dye deoxy terminator sequencing kit after digestion with T7 gene 6 exonuclease. In (b) the results of the reverse sequencing of the same product are represented using Taq Dye deoxy terminators without the use of T7 gene 6 exonuclease, i.e. using double-stranded PCR product as template for cycle sequencing. Sequencing is performed using the antisense primer as sequencing primer. The results obtained with Taq Dye deoxy terminators show a positive peak for all different nucleotides with an extremely low background signal, close to baseline, for the other three nucleotides (see figure 3a and 3b). These good quality data allow reliable detection of heterozygosity in PCR products after 15 minutes digestion with T7 gene 6 exonuclease, as is shown for base 1 of codon 12 of K-ras, where 2 clear signals are present. This base normally shows a G coding for the wild type in the sense strand and a C in the antisense strand. With the expected G to T transversion in this position, the corresponding antisense strand shows the A signal for this base. On the contrary, only the dominating allele (T) is detected for base 1 of codon 12 of K-ras using double-stranded PCR products for cycle sequencing (b). This is in agreement with the results described before, only detecting the dominating allele for cell line Calu-1 using the Point-EXACCT and the Sanger sequencing method¹⁸⁷. Since the sensitivity of the Point-EXACCT method was not optimized at that point, only the dominating allele (T) could be detected, whereas after optimization of the Point-EXACCT procedure consistent reliable detection of heterozygosity for base 1 was obtained. The sensitivity of the originally used Sanger sequencing method is not sufficient to reliably detect heterozygosity.

The results after the reverse sequencing of the 233-bp fragment of the K-ras gene from a patient with an adenocarcinoma of the lung are represented in figure 4. This patient contains both wild type (G) and mutated (T) sequence for base 1 of codon 12 of the K-ras gene, detected by Point-EXACCT (data not shown). For this patient, both wild type (G) and mutated (T) sequence were detected using single-stranded template for sequencing with either Taq Dye deoxy terminators (see figure 4a), while only the wild type sequence (G) is detected for base 1 of codon 12 of K-ras using double-stranded PCR product sequencing (see figure 4b). The exonuclease-enhanced sequencing method was further tested on a series of adenocarcinomas of the lung, of which 3 out of 10 were clearly positive for K-ras codon 12 base 1 (an example is shown in figure 4a).

Figure 3. The results after the reverse sequencing of the 233-bp fragment of the K-ras gene from cell line Calu-1 using (a) Taq Dye Deoxy terminators after the use of T7 gene 6 exonuclease. In (b) the results of the reverse sequencing of the same product are represented using Taq Dye Deoxy terminators without the use of T7 gene 6 exonuclease, i.e. using double-stranded PCR product as template for cycle sequencing. The four different nucleotides are indicated by G (guanosine), A (adenosine), T (thymidine) and C (cytosine). Codon 12 base 1 is marked with an arrow. Both C (full peak) and A (dashed peak) sequence, corresponding to forward wild type (G) and mutated (T) sequence for base 1 of codon 12 of the K-ras gene were detected using Taq Dye Deoxy terminators (a) after digestion with T7 gene 6 exonuclease, while only the dominating allele (T) was detected for K-ras codon 12 using double-stranded PCR products for cycle sequencing (b). (Note that the sequenced strand (reverse) data are displayed from 5' to 3' direction).

Fig. 4. The results of the 233-bp fragment of the K-ras gene of a lung cancer patient, after reverse sequencing with Taq Dye Deoxy terminators. Both C and A sequence (full peak and dashed peak), denoting forward wild type (G) and mutated (T) sequence for base 1 of codon 12 of K-ras were detected after digestion with T7 gene 6 exonuclease. Only the peak coding for cytosine and corresponding to the forward wild type sequence (G) was obtained after double-stranded PCR product sequencing (b).

The exonuclease-enhanced fluorescent sequencing method is based on a secondary PCR approach to join phosphorothioate (S)-linkages to one strand

of the amplification products. However, S-linkages can also be directly incorporated without the use of a secondary PCR approach: incorporation of S-linkages into one of the amplification primer apparently blocks digestion of that strand completely, so that the desired single-stranded amplification product can be obtained after digestion with T7 gene 6 exonuclease. T7 gene 6 exonuclease is active in the same buffers used for PCR; thus, amplification and exonuclease digestion can sequentially be done in the same reaction tube. This simple use of exonuclease pretreatment of single-stranded amplification products, also efficiently eliminating byproducts which could impede the cycle-sequencing reaction, produced highly efficient quality of sequencing data, resulting in reliable detection of heterozygous sequence differences. Furthermore, due to the use of cycle sequencing, only a limited amount of amplification products is required for the start of the sequencing reaction. Using Taq terminators for direct cycle-sequencing, the need for special primers for the sequencing reaction is eliminated, since the antisense primer of the amplification reaction could be used as sequencing primer.

A potential shortcoming of the use of Taq DNA polymerase for cycle-sequencing could be the fact that variable peak intensities could occur due to uneven incorporation of the different dideoxynucleotides. This could cause problems with the "base calling" ability of the computer software resulting in the assignment of one peak to a single particular base rather than to designate it as an "N" to indicate heterozygosity. Hence, this can be prevented by careful observation of the raw sequencing data. Furthermore, since the use of Sequenase gives much more uniform peaks¹⁸⁴, we also performed the same experiments with a thermostable Sequenase. However, the detection of heterozygote sequence differences in those cases could not clearly be confirmed.

The second method based on exonuclease pretreatment of amplification products is point mutation detection by Point-EXACCT. A serial dilution of the enzyme T7 gene 6 exonuclease ranging from 0.03-4U/ μ l was used for determination of exonuclease conversion to single-stranded DNA with Point-EXACCT. Even small amounts of the enzyme (0.03U/ μ l) effectively converted amplification products into a detectable form, whereas higher amounts (0.3 to 3U/ μ l) resulted in more sensitive detection of point mutations. The amplified products digested with 3U/ μ l exonuclease concentration provided the highest optical density signal, resulting in the most sensitive detection of point mutations. Digestion with 4U/ μ l exonuclease did not improve sensitivity. The hybridization efficiency after heat-denaturation alone (without exonuclease digestion) resulted in 60-70% of the relative optical density values obtained from exonuclease-treated products (see figure 5). Washing procedures must be carefully carried out, reducing the risk of carry over. Presently, we use every other row in the microtiter plate for washing alone. After eliminating the washing substrate from one well, the pipette is washed with an equal volume PBS/0.05% Tween 20. Repeating the same sample 10 times yielded a coefficient of variation in relative optical density of less than 3%.

Figure 5. Detection of point mutations in exonuclease-digested PCR products by Point-EXACCT compared to double-stranded PCR products. The relative optical densities obtained after heat-denaturation (black bars) and exonuclease digestion (checked bars) are shown for cell line HL60 and a dilution 1/3,125 of cell line A549 containing a K-ras mutation in wild type cell line HL60. Optical density from the ligated products is expressed relative to the (maximal) optical density of the controls: a hybridized sample without ligation. The same volumes of amplification products were processed simultaneously for heat-denaturation and exonuclease digestion. Detection of single-stranded products was greater compared to double-stranded products when the same volumes of samples were used.

After the optimization of the different parameters affecting the Point-EXACCT method, the sensitivity was shown by reconstruction experiments in which vital cells bearing a K-ras codon 12 mutation were mixed with vital wild type cells. For this experiment a serial dilution (1:5) was made for both cell lines A549 and Calu-1 with wild type HL60. A decrease in the relative optical density was obtained after hybridization with the probe directed against the specific mutation with increasing dilutions of the cells with a K-ras mutation to wild type cells (figures 6). Using 1 μ g DNA in 100 μ l for the amplification reaction, the relative optical density signal of dilution 1:15,000 was still above the background, demonstrating the detection of 1 cell containing a K-ras mutation in an excess of 15,000 wild type cells after exonuclease pretreatment (figure 6a and 6b). The threshold line of the background values was set at the mean plus three times the standard deviation. For cell line A549 and cell line Calu-1 the threshold percentages are 10.5% and 8.0%, respectively. For these values the one sided maximal risk of false positivity is 0.5%. For higher relative optical density values this chance on false positivity

reduces rapidly more. Since Point-EXACCT uses 8.5 μ l amplification product after a PCR reaction containing 1 μ g DNA in 100 μ l amplification reaction mix, and assuming a DNA content of 5-7 pg per cell, approximately 12,000-17,000 cells are present at the start of the Point-EXACCT hybridization reaction per well.

Reconstruction experiment

a) A549/HL60

Reconstruction experiment

b) Calu-1/HL60

Figure 6. Reconstruction experiments after serial dilutions of vital for K-ras codon 12 and wild type cell line and testing with Point-EXACCT using $1\ \mu\text{g}$ DNA in $100\ \mu\text{l}$ for the amplification reaction. The results of the reconstruction experiments are shown for A) cell line A549 and B) cell line Calu-1. In this figure, the optical densities obtained from the different dilutions of the cell line containing a K-ras mutation in the wild type cell line HL60 were expressed relative to the (maximal) optical density of the undiluted products. The relative optical density values above the threshold values are called positive. The threshold value (represented by the broken line) is obtained by the mean plus $3\times$ standard deviation of the relative optical density from the ligated biotinylated control probes. For A549 and Calu-1, these values are 10.5 and 8.2, respectively.

In an attempt to investigate the maximum sensitivity, up to $4\ \mu\text{g}$ DNA was used in the amplification reaction. Using this high concentration of DNA a signal above the background (10.0%) could be obtained for A549 at the 1:78,000 dilution, demonstrating the detection of 1 cell containing a mutation amidst more than 75,000 wild type cells. For Calu-1 the relative optical density signal is close to the threshold line at this dilution (8.1% of control without ligation) (data not shown). For the converse experiment, the same results were obtained, confirming the high sensitivity of Point-EXACCT. Theoretically in this setup DNA of about 50,000 cells is present in each well. In practice 1 cell containing a mutation amidst 75,000 and 15,000 wild type cells could be detected in cell lines A549 and Calu-1, respectively. Thus, using exonuclease a high sensitivity can be obtained.

Reproducibility is high, since five times repeating of the reconstruction experiments, after continued cell cultures yielded the same results.

The Point-EXACCT method is rapid. Starting from genomic DNA obtained from either paraffin or frozen sections, blood cells, fecal material, bronchial epithelial cells, or sputum, the detection of point mutations can be accomplished within 10 hours. Therefore, analysis of point mutation detection by Point-EXACCT requires considerably less time and effort than other techniques. Another advantage of Point-EXACCT is that only small numbers of cells of DNA samples are sufficient for analysis. Therefore, this method is useful for microdissection studies as well.

The oligonucleotide ligation assay was developed to circumvent the need for electrophoresis and critical hybridization conditions. The ligase enzyme is, like in Point-EXACCT, associated with a high specificity. The time for oligonucleotide ligation assay is similar as the time needed for Point-EXACCT. However, in the oligonucleotide ligation assay the sensitivity is lower, since the procedure uses heat denaturation. Using T7 gene 6 exonuclease increased the hybridization efficiency compared to heat-denatured amplification product alone. The signal difference expressed in relative optical density corresponds to a factor of 10-100 in cell number, demonstrating the improvement of solution hybridization after exonuclease pretreatment.

Essential in the Point-EXACCT method is that (i) only molecules hybridized with two adjacent probes to single-stranded DNA, (ii) which are

subsequently ligated, will be detected by this format ensuring a very high degree of sensitivity and specificity. These characteristics make this technique highly suitable for screening purposes, where frequently less than 1 malignant cell is present in 100-1000 normal cells. Potential (pre)clinical screening applications can be sputum or washings obtained during bronchoscopy from patients with lung cancer, fecal material from patients with colon cancer and urine from patients with bladder cancer. The simplicity, reproducibility, high specificity and sensitivity of Point-EXACCT makes it a highly promising method for large-scale cancer screening.

In conclusion: exonuclease-enhanced PCR sequencing results in high quality of sequencing allowing detection of heterozygosity. With this rapid, simple and efficient improved method for detecting heterozygosity, the detection of point mutations using fluorescent cycle sequencing is becoming more reliable. This sequencing method can well be used for detection of sequence mutations in genetic, infectious and neoplastic diseases. The high sensitivity of the Point-EXACCT method due to the use of exonuclease makes it useful for detection of early occurring point mutations such as K-ras in a heterogeneous cell population, where only a small fraction of tumor cells containing a mutation is present among much larger number of normal cells.

Four

Validation of Point-EXACCT

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*Veerle AMC Somers, Darcy A Leimbach, Paul HMH Theunissen,
James J Jr Murtagh, Brian Holloway, Anton W Ambergen
and Frederik BJM Thunnissen*

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Abstract

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.

1. Introduction

Point mutations in the Kirsten ras (K-ras) oncogene are one of the most common genetic alterations involved in various types of human cancer^{88,89}. In lung cancer, K-ras mutations occur predominantly in codon 12^{80,84,93-95}. 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 (NSCLC)^{80,81,93,95-100,104}. 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^{92,105,106}, 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^{57,129}.

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^{13,17,80,81,84,97,111-113,173}. At the moment, a number of methods displaying considerable increase in sensitivity of point mutation detection have been described. Those methods include amplification refractory mutation system¹⁸⁹, allele-specific amplification¹⁹⁰, mismatch amplification mutation assay¹⁹¹, oligonucleotide ligation assay¹⁸⁶, ligation chain reaction¹⁹², restriction fragment length polymorphism¹⁹³, enriched PCR¹⁹⁴, PCR-primer-introduced-restriction analysis with enrichment of mutant alleles¹⁹⁵, PCR-based cloning and hybridization¹⁹⁶ and point mutation

detection using exonuclease amplification coupled capture technique (Point-EXACCT)^{187,197}. Interestingly, in those studies the percentage of cases positive for K-ras seems 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 mostly 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%¹⁹⁵. 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^{99,195,198,199}. Because K-ras mutations can be present in only a subpopulation of the tumor cells⁹⁹, 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^{187,197}. 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.

2. Materials and Methods

2.1 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-eosin stained sections. Histopathologic classification was performed according to the World Health

Organization guidelines²⁰⁰ on formalin-fixed, paraffin embedded and subsequently hematoxylin-eosin stained slides. DNA was isolated from 25 micrometer sections of tissue blocks using a rapid lysis procedure¹⁹⁷. The study protocol was approved by the local ethical committee.

2.2 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^{88,188}; A549, which has a homozygous serine mutation (AGT)¹⁸⁰; and Calu-1, which has a heterozygous cysteine mutation (TGT)¹⁸¹. SW480 is homozygous for the K-ras codon 12 valine mutation (GTT)¹⁸¹.

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 before¹⁹⁷. Reconstruction experiments for codon 12 base 1 and 2 were performed with cell lines A549 and Calu-1 and SW480, respectively.

2.3 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 previously¹⁹⁷. 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 base 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 μ l of 1 x ligase buffer (Promega Corporation, Madison, WI), 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 revealed a 233 bp product using a secondary PCR approach with the same thermal cycling profile as described before¹⁹⁷.

For fluorescent cycle sequencing, amplification products were digested with exonuclease I (United States Biochemical, Cleveland, OH) 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 products¹⁹⁷. 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 K18¹⁹⁷. 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.

3. Results

3.1 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-eosin stained frozen sections of the tissue block. For the tumor part and normal components, these values were $45 \pm 15\%$ (range 5-80%) and $37 \pm 14\%$ (range 10-90%), respectively. Furthermore, for the necrotic cells, these values were estimated to be $18 \pm 19\%$ (range 0-90%).

3.2 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, base 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 optical density 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 (1/3,000 and 1/15,000) represent positive controls, leading to positive signals for the specific mutation after hybridization and ligation with probe T.

As external negative controls for base 1, relative optical density 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, base 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 base 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, that produced 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).

Table 1. Distribution of K-ras mutations found with Point-EXACCT by histological subtype

histology	AGT	TGT	CGT	GAT	GTT	GCT	ATT ^a	TOTAL
SQCC	1	0	0	0	7	0	0	8
adeno	0	2	0	2	5	0	1	10
LCC	1	1	0	0	0	0	1	3
TOTAL	2	3	0	2	12	0	2	21

^a Note that ATT represents a double mutation

Because Point-EXACCT is a colorimetric assay, a level of positivity can be discerned. Relative optical density 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 optical density signals obtained from several reconstruction experiments¹⁹⁷, this 30% threshold signal corresponded to a mutation fraction between 1/625 and 1/3,125. 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).

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

histology	high fraction	low fraction	TOTAL
SQCC	1	7	8
adeno ^a	6	4	10
LCC ^a	2	1	3
TOTAL	9	12	21

^a Note that one patient with a LCC and another patient with an adeno contained one double mutation, which both referred to high fraction mutations

3.3 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.

3.4 Sequencing of single-stranded amplification products

The use of T7 gene 6 exonuclease yields highly efficient data for sequencing¹⁹⁷. 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.

4. 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 prior to direct cycle sequencing, producing increased hybridization efficiency of the sequencing primer to the single-stranded amplification products, as reported previously¹⁹⁷. The results after exonuclease digestion demonstrated that all cases with a mutation identified by single-stranded sequencing were also positive for Point-EXACCT. No discordances 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^{104,201,202}. The 35% frequency of K-ras mutations in adenocarcinomas coincides with several other reports^{104,201}, but a much higher frequency was found in our study for large cell carcinomas compared with others^{96,201}. Furthermore, the 15% frequency of cases positive in squamous cell carcinomas is the same as found in several other studies^{199,201} 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^{80,96}.

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 wildtype glycine was changed most often to valine^{84,97,101,113,199}.

With regard to the fact that low fraction mutations revealed by Point-EXACCT 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^{99,199,203}, 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 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 coworkers^{199,204} 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 al¹⁹⁵ 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^{205,206}. However, this risk may be reduced by using other polymerases with greater fidelity²⁰⁷. An adaption of the method described by Mills et al.⁹⁹, 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.

Five

K-ras detection in sputum

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Detection of K-ras point mutations in sputum patients
with adenocarcinoma of the lung

*Veerle AMC Somers, Alexandra M Pietersen, Paul HMM Theunissen,
and Frederik BJM Thunnissen*

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Abstract

Kirsten (K-ras) point mutations are found in 30-56% of pulmonary adenocarcinomas by means of highly sensitive techniques. Recently, the Point-EXACCT (point mutation detection using exonuclease amplification coupled capture technique) method was described, which detected one cell with a mutation in 15,000 normal cells. The aim of this study was to examine whether K-ras point mutations could be found with this rapid method in sputum of patients with adenocarcinoma of the lung.

DNA from paraffin-embedded adenocarcinoma and corresponding sputum samples were analyzed for mutations of the K-ras gene. Twenty-eight biopsy and 54 sputum samples of 22 patients were used for amplification and K-ras codon 12 point mutation detection.

In 11 of 22 patients (50%), a mutation in K-ras codon 12 was shown in the tumor sample. In 5 of 11 patients (45%) with a K-ras mutation in the tumor, the same type of mutation was identified in at least one sputum sample. A mutation could not be detected in any of the sputum samples from patients with a K-ras negative tumor. Time between K-ras point mutation detection in sputum and clinical diagnosis of lung cancer varied from one month to almost 4 years. In two of the five patients with K-ras-positive sputum specimens, malignant cells were found with cytological examination.

Point-EXACCT is suitable for detection of K-ras point mutations in sputum samples of patients with adenocarcinoma of the lung. This approach may be an important adjunct to cytology in the early diagnosis of lung cancer.

1. Introduction

Lung cancer is a disease with high incidence and mortality²⁰⁸. The prognosis for patients with lung cancer is most favorable when tumors are detected early in a surgically resectable stage of non-small cell lung cancer (NSCLC). Methods that can increase the percentage of cases of lung cancer detected in early stage may theoretically lead to a decrease in mortality. However, in the past, screening programs for early detection of lung cancer that used chest X-ray of the thorax with or without sputum cytology have not been successful in reducing the high numbers of lung cancer deaths¹⁶⁵⁻¹⁶⁸. Therefore, new approaches that use genetic alterations as potential biomarkers may be beneficial for early detection.

Genetic alterations, such as point mutations in the Kirsten (K-ras) oncogene, have been suggested to have a role in the multistep process of lung carcinogenesis. Among different histologic NSCLCs, K-ras point mutations have been detected with variable frequency in adenocarcinomas, but since the

use of highly sensitive detection methods, point mutations were also found in squamous cell carcinomas, although to a limited extent^{104,209}.

At the moment, a number of reports have described the identification of point mutations in several exfoliated cells into which tumor cells are often shed from the tumor. Sidransky et al^{210,211} first showed K-ras point mutations in exfoliated cells in the stool of patients with resectable and potentially curable colon cancer and the identification of p53 mutations in urine obtained during surgery in patients with bladder cancer. Another group was successful in the detection of those mutations in pancreatic juice and peripheral blood of patients with pancreatic adenocarcinoma²¹².

In lung cancer, Mao et al¹⁹⁶ first described identification of K-ras point mutations in tumor and corresponding initially stored sputum specimens from patients who later developed adenocarcinoma of the lung. Furthermore, several other groups have shown the feasibility of the detection of K-ras codon 12 mutations in individuals suspected of having lung carcinoma by point mutation analysis in sputum or bronchoalveolar lavage fluids by using sensitive techniques that enable detection of those genetic alterations when present in a low fraction of the genetically normal cells^{100,192,203,213-215}. These methods include mutant-allele-specific amplification²¹⁴, polymerase chain reaction (PCR)-primer-introduced restriction analysis with enrichment of mutant alleles (PCR-PIREMA)²⁰³, enriched amplification methods such as enriched PCR^{100,202} and enriched single-strand conformational polymorphism²¹⁵, allele-specific hybridization after cloning^{196,216} and PCR-based ligase chain reaction (LCR)¹⁹².

We have developed a highly sensitive method for the detection of known point mutations, Point-EXACCT (point mutation detection using exonuclease amplification coupled capture technique), which enables the detection of one cell that contains a mutation in more than 15,000 wild type cells^{187,197,217}. The aim of this study was to examine with this rapid assay whether K-ras point mutations could be detected in sputum of patients with adenocarcinoma of the lung.

2. Materials and Methods

2.1 Patients and tissues

Using PALGA (the Dutch national pathology-coding computer network system), records of patients who underwent lobectomy or pneumectomy for NSCLC between 1983 and 1993 were retrospectively selected from the archives of the De Wever Hospital (Heerlen, The Netherlands). Patients diagnosed with adenocarcinoma were chosen because this subtype of NSCLC shows a high prevalence of K-ras point mutations^{99,196}. Only those patients from whom at least one sputum sample collected before histologic diagnosis

was available were designated. This resulted in a group of 22 patients of 88 patients with adenocarcinoma of the lung who underwent resection with curative intent at De Wever Hospital between 1983 and 1993 (data from Maastricht Cancer Registry).

Twenty-eight biopsy specimens and 54 sputum samples of 22 patients were used. On average, 2.5 sputum samples were available per patient (range, one to seven samples). The patient population consisted of 20 men (age range, 41 to 88 years) and 2 women (43 and 44 years of age). The files could be retrospectively retrieved for 11 of 22 patients. All 11 patients were heavy smokers with more than 40 pack-years verifiable in eight of them. Staging information was based on file and pathology reports, but in two patients, the retrospective staging data were insufficient for reliable classification. According to the 1978 staging system, the distribution for stage I, II and III was 12, four and four patients, respectively²¹⁸. According to the 1986 staging system, the stage distribution was I, II, IIIa, and IIIb, with 11, five, two and two patients, respectively²¹⁹.

Biopsy or resection samples, preserved in archival paraffin-embedded tissue blocks were analyzed. Histopathologic classification was performed after hematoxylin and eosin staining of formalin-fixed tissue sections using the World Health Organization criteria²⁰⁰.

Stored (noninduced) sputum was retrieved, which after collection had been fixed in alcohol, embedded in paper, and transferred to paraffin overnight. From the paraffin blocks, 5- μ m sections were cut, stained with hematoxylin and eosin, and used for cytologic diagnosis. The remainder of the sample in the paraffin block was used for K-ras analysis in the present study.

2.2 DNA isolation and K-ras gene exon 1 amplification

Paraffin-embedded blocks of biopsy and sputum samples were dewaxed in xylene and rehydrated. DNA was isolated by using proteinase K digestion and used for amplification with primers of the first exon of the K-ras gene outside the codon 12 region, as previously described¹⁹⁷.

DNA extracted from several cell lines with wild type or mutated sequences for K-ras codon 12 served as controls in the Point-EXACCT method. HL60 and NCI-H716 contain the wild type DNA for K-ras codon 12 (GGT)^{188,189}; A549, a homozygous serine mutation (AGT)¹⁸⁰; and Calu-1, a heterozygous cysteine mutation (TGT),¹⁸¹ respectively. SW480 is homozygous for the K-ras codon 12 valine mutation (GTT), and SW1116 contains an alanine mutation (GCT) for codon 12 of the K-ras gene¹⁸¹.

Concerning the sensitivity of Point-EXACCT, several reconstruction experiments were made in which vital cells that bear a K-ras codon 12 mutation were serially diluted in wild type cells, as previously described¹⁹⁷.

Reconstruction experiments for codon 12 base 1 and 2 were performed with cell lines A549, Calu-1, and SW480, respectively.

2.3 K-ras amplification and point mutation detection

Conditions for K-ras amplification leading to 204 bp product and subsequent point mutation detection by Point-EXACCT have been previously described in detail¹⁹⁷. Both biopsy and sputum samples were subjected to amplification and point mutation analysis by Point-EXACCT at least twice. Point-EXACCT was performed with probes directed against base 1 and 2 of K-ras codon 12. A slight modification of the Point-EXACCT method was made¹⁹⁷, using 1.6 pmol/L 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 μ L of 1 x ligase buffer (Promega Corporation, Madison, WI), which consisted of 30 mmol/L of Tris-HCl, pH 7.8; 10 mmol/L $MgCl_2$; 10 mmol/L dithiothreitol and 0.5 mmol/L Adenosine 5' Triphosphate without the addition of formamide.

Amplification and point mutation detection by Point-EXACCT included multiple positive and negative controls in each experiment. Amplification products that contained a known mutant fraction of 1/15,000 were used in each experiment as positive controls for the sensitivity. Furthermore, all samples were separately tested with each of four different base specific probes (G, A, T and C). As an external positive control with a mutation for base 1 of K-ras codon 12, cell lines A549 and Calu-1 were used, which give relative optical density signals for the specific mutation after hybridization and ligation with probe A and T, respectively. For base 2 of K-ras codon 12, dilutions 1/3,000 and 1/15,000 of cell line SW480 in the wild type cells HL60 were used as positive controls, which resulted in positive signals for the specific mutation after hybridization and ligation with probe T.

Relative optical density values of negative probes (background values) T and C and A and C of the cell lines A549 and Calu-1 were used as external negative controls for base 1. Values obtained from probes A and C on the SW480 mixture give background values for base 2 of K-ras codon 12. For each specific nucleotide of K-ras codon 12 base 1 and 2, the mean, SD, and threshold values were calculated based on corresponding background values. Overall, the threshold value based on negative control probes for each specific nucleotide is calculated by mean plus three times the SD of the background values. Therefore, 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 is called positive (i.e., mutation present) in the Point-EXACCT assay when duplicate signals for a specific nucleotide exceed the threshold value for the corresponding nucleotide based on the negative and, if available, positive control for base 1 and 2 separately.

2.4 Statistical analysis

The χ^2 analysis was performed to test the association between presence of mutations in sputum of patients with and without K-ras-positive tumor specimens.

3. Results

The Point-EXACCT method was used for analysis of K-ras codon 12 alterations in biopsy and/or resection samples from patients with adenocarcinoma of the lung and the corresponding sputum samples. All tumor and sputum samples that were collected before clinical diagnosis of lung cancer were analyzed in a blinded fashion for K-ras point mutation detection with Point-EXACCT.

The 28 tumor samples included 18 resection specimens, nine biopsy specimens (biopsy locations: of bronchus, lung, and pleura), and one lymph node metastasis specimen. The number of K-ras point mutations found in tumor and sputum specimens is shown at patient and sample levels in table 1. K-ras codon 12 alterations were found in 15 of 28 (54%) tumor samples, whereas 45% of K-ras-positive tumors were also positive in sputum samples (table 1). Forty-four percent of the mutations identified in biopsy and tumor specimens from surgical resection were G to A transitions, whereas 56% of the mutations represent G to T transversions.

Table 1. Frequency of K-ras codon 12 mutations in tumor and sputum specimens of patients with adenocarcinoma of the lung.

	Total	K-ras-Positive	
		No.	%
Patients			
Tumor	22	11	50
Sputum	22	5	23
Samples			
Tumor	28	15	54
Sputum	54	6	11

From six of 22 patients, in time two different tumor specimens obtained from biopsy and surgical resection were analyzed, which showed the same outcome in four of six patients. In one of the remaining two patients, a serine and cysteine (AGT and TGT, respectively) mutation was identified in the specimen obtained after resection, whereas a biopsy specimen collected 1 month later only showed a cysteine mutation. The other remaining patient first presented with an adenocarcinoma in the left upper lobe. Point mutation analysis of this tumor after resection identified an aspartic acid mutation (GAT) for K-ras codon 12. Two years later, this patient presented with another tumor mass in the right lung, histologically classified as a highly differentiated adenocarcinoma, which contained a cysteine mutation (TGT) for K-ras codon 12.

Table 2 lists the results of sputum samples that corresponded to positive tumor specimens obtained with Point-EXACCT and previous routine cytology. In five patients with a K-ras-positive adenocarcinoma, the identical mutation in the tumor was also detected in sputum. Furthermore, no differences in the type of mutations were shown, except for one patient. This patient (patient no. 12; table 2) had a serine and cysteine mutation (AGT and TGT), whereas both sputum specimens from this patient only showed a serine mutation (AGT). In addition, no other point mutations were detected in the sputum samples, which indicates the high specificity of the Point-EXACCT method. The χ^2 test for the association between the presence or absence of mutations in the sputum and corresponding tumor specimens showed a significant difference ($P = .011$).

Table 2. Results of K-ras codon 12 point mutation analysis by Point-EXACCT (PE) and routine cytology in tumor and sputum specimens

Patient No.	K-ras Tumor	K-ras Sputum	No. of ^a PE	Time of sputum ^b collection		Cytology ^c
				Positive	Range	
1	GAT	GGT ^d	0/1	-	27	NA
6	AGT	AGT	1/3	1,413	1,411-1,415	-
7	AGT	AGT	1/6	20	12-20	-
8	TGT	GGT	0/3	-	6	-
9	TGT	TGT	1/1	15	15	+ ^e
10	TGT	TGT	1/7	98	10-130	-
12	AGT/TGT	AGT	2/2	58	58	+ ^e
13	GAT	GGT	0/3	-	1,198-1,502	-
	TGT	GGT	0/3	-	1,818	-
17	AGT	GGT	0/1	-	11	NA
20	GTT	GGT	0/1	-	10	-
21	TGT	GGT	0/3	-	11-12	-

^a Number of sputum specimens positive for K-ras codon 12/total number of sputum specimens for this patient with Point-EXACCT (PE)

^b Time in days before final tumor diagnosis and time of first sputum specimen positive by Point- EXACCT is given.

^c Positive or negative for malignancy in original cytological investigation; NA, specimen could not be diagnosed in original cytological investigation

^d GGT refers to wild type sequence for K-ras codon 12

^e For patients no. 9 and 12, malignant cells were found in one of one and one of two sputum samples with cytology, respectively.

In most of the patients with a K-ras mutation in the tumor, only one of several different sputum specimens was found to have the mutation. For the 54 sputum specimens examined, the mean time between sputum collection and histologic diagnosis of the tumor was 11 months (range, 0 to 60 months). The mean time for K-ras-positive tumors and negative sputum and positive tumor and sputum specimens was 15 months (range, 0 to 60 months) and 9 months (range, 0.5 to 46 months), respectively. This difference was not significant ($P = .44$).

The earliest detection of a clonal population of cells that contained a mutation in at least one sputum sample that corresponded to the tumor was

obtained 46 months before histologic diagnosis of the tumor (patient no. 6; table 2). With cytologic examination of three different sputum specimens in this patient, malignant cells were not diagnosed.

Tumor specimens obtained from patients diagnosed with an adenocarcinoma of the lung showed high-fraction mutations (data not shown), whereas point mutations identified in corresponding sputum samples showed low-fraction mutations, which referred to the presence of only a minority or a single cell with a mutation among an excess of normal cells. Based on the results of several reconstruction experiments with control cell lines, the mutation fraction showed in one sputum specimen was higher than 1/3,125. Relative optical density signals from three K-ras-positive sputum samples corresponded to a dilution 1/3,125, and for three other K-ras-positive sputum samples, relative optical density signals were similar to 1/15,000.

Using Point-EXACCT, we were able to detect K-ras codon 12 alterations in patients who were diagnosed negative for malignancy in the original cytological investigation. Only two of five patients with K-ras-positive sputum were initially diagnosed positive for malignancy with cytologic investigation. The remaining patients were all diagnosed negative by routine cytology.

In addition, for six of 54 samples, cytologic examination was not possible because of insufficient quality of the cytologic material. In two of those six specimens, K-ras point mutations were detected (table 2).

4. Discussion

The purpose of this study was to examine K-ras point mutations in sputum specimens obtained from patients with adenocarcinoma of the lung. Using Point-EXACCT, point mutations in K-ras codon 12 were identified in 50% of tumor samples, and five of 11 positive tumor samples were also found positive for the same K-ras codon 12 alteration in sputum specimens collected before clinical diagnosis.

The 50% frequency of K-ras codon 12 alterations shown in tumor specimens from patients with adenocarcinoma of the lung corresponds to the results reported by other groups that used highly sensitive methods for detection²⁰³. Despite the fact that the sputum material had been stored for more than 10 years, we were able to perform K-ras point mutation analysis on specimens that could not be previously diagnosed with routine cytology. Moreover, we were able to detect the same K-ras codon 12 alterations in sputum samples of 23% of patients with adenocarcinoma and further show point mutations in sputum specimens that were identified as without malignancy by routine cytology. This higher number of mutations found with Point-EXACCT compared with routine cytologic diagnosis is explained by the high sensitivity of this molecular method¹⁹⁷.

The present study also showed that K-ras codon 12 mutations were identified in a very low percentage of cells in sputum specimens of patients with lung cancer. Because multiple control samples with known mutant fractions were analyzed in each experiment, we could determine the mutation fraction in both tumor and sputum specimens. As a result, a sample sensitivity for mutational detection between 1/3,000 and 1/15,000 was achieved in the majority of sputum specimens. These results indicate that Point-EXACCT is able to discern the presence of a single cell that contains a mutation among the majority of genetically normal cells.

From patients in whom different tumor specimens in time were obtained (biopsy specimen and corresponding tumor from surgical resection), the same type of mutation was identified in both specimens, which confirms the reproducibility of the point mutation method. Furthermore, identical K-ras mutations were found in sputum and corresponding tumor samples. Patients with tumors that only showed genetically normal sequence for K-ras codon 12 also had negative sputum samples, which confers to high specificity of the detection method. Furthermore, independent amplification and point mutation detection reactions showed the same outcome. Altogether, these findings affirm the high sensitivity, reproducibility and specificity of this point mutation detection method.

In five of 11 (45%) patients with K-ras positive adenocarcinomas, the same mutation was found in sputum specimens. The following explanation can be offered for this relatively low percentage: (1) The K-ras-negative samples do not contain tumor cells. The material investigated was the remainder of paraffin blocks after cytologic investigation was initially performed. Possibly, tumor cells may have been present initially, but were cut out of the paraffin block at the time of cytologic diagnosis. Alternatively, no tumor cells were present in the sputum at all. Previously, when sputum cytology was used for detection of malignant cells, the importance of sampling more sputum specimens was reported^{220,221}. In patients with primary lung cancer, the proportion of correct positive cytological diagnoses increased from 47% to 87% by examining one up to five sputum specimens²²⁰. Furthermore, in patients who produced bloody sputum, the examination of at least three different sputum samples increased the sensitivity of cytologic diagnosis²²¹. This problem of limited shedding of cells may possibly be overcome by the examination of 3-day pooled collections of sputum specimens or the use of induced sputum. (2) Another explanation for the 45% frequency in sputum specimens of all K-ras-positive adenocarcinomas is that tumor cells were present in residual cells in paraffin blocks, but at less than the detection level of the Point-EXACCT method.

The results of the first retrospective study that examined tumor specimens and corresponding stored sputum samples obtained before clinical diagnosis for the presence of K-ras or p53 mutations were reported by Mao et al¹⁹⁶. The earliest detection of a clonal population of cancer cells in sputum

was in a sample obtained more than 1 year before clinical diagnosis. In our study using Point-EXACCT, the time between K-ras positivity in sputum and the first date of histologic diagnosis varied between 1 month to almost 4 years (46 months), which indicates that a time period may exist for opportunity for early detection. In general, it appears that there is a period of 1 to 10 years during which individuals who develop bronchogenic carcinoma exfoliate cells in their sputum, but have no clinical symptoms²²². This interval, which may average 5 or more years, appears to be long enough to permit early detection and result in improved therapy²²². Therefore, periodic sputum analysis of groups at elevated risk of NSCLC can use this period for early detection and treatment.

Because adenocarcinomas occur in the periphery of the lung, it can be envisaged that tumor cells are less likely to be sampled in the sputum from the outer parts of the lung without induction. Nevertheless, with the use of noninduced sputum in the present study, in 45% of K-ras-positive tumors, point mutations in corresponding sputum samples were found. This was lower than that reported by Mao et al, who used sputum specimens induced by inhalation of hypertonic salt solution¹⁹⁶.

From patient no. 7 (table 2), we retrieved six different sputum samples collected before clinical diagnosis of the tumor. None of those samples was positive for malignancy by cytologic analysis, whereas Point-EXACCT was able to detect the same point mutation that corresponded to the tumor in at least one sputum sample. This patient was further treated by a lung wedge excision. Remarkably, this patient had a positive sputum sample 14 months after resection of the tumor. On further examination, a local recurrence was present. Patient no. 12, with a resection of both right middle and upper lobes, showed a double mutation for base 1 of codon 12, which represented both AGT and TGT mutation. Histologic diagnosis showed an adenocarcinoma of the lung with a combined pattern. The peripheral part of this adenocarcinoma was moderately differentiated, whereas the central part showed an undifferentiated pattern. From this patient, we retrieved 2 different sputum samples that were collected 2 months before resection of the tumor. Cytologic diagnosis only identified one sputum sample with an adenocarcinoma, whereas both samples were positive for K-ras codon 12 alterations. After 1 month, the local recurrence of the tumor was confirmed by histopathologic diagnosis. An analysis of the bronchial biopsy specimen showed again a TGT mutation for K-ras codon 12.

Point mutation detection in K-ras codon 12 in sputum of patients with NSCLC may be an important approach for early lung cancer detection because recently it has been shown in animal studies^{105,107} that those mutations occur before the macroscopical appearance of the tumor. It is, therefore, important to look at the initiation stage of the tumor for those mutations in a relatively early clone of cells with a K-ras mutation that progress to a malignant tumor. Mills et al¹²⁰³ have reported the occurrence of K-ras codon 12 alterations in

bronchoalveolar lavage fluid of patients with highly suspected lung cancer. Compared with the use of bronchoalveolar lavage fluid in lung cancer diagnosis, the possibility of detecting K-ras point mutations in sputum specimens offers great benefits for the patient because it is less invasive. Because all lung cancer is believed to develop from bronchial epithelial cells, sputum remains an attractive potential vehicle for early detection and for the use of genetic alterations, such as K-ras, in the multistep process of lung carcinogenesis.

Point-EXACCT has several advantages compared with previously described highly sensitive methods used for K-ras point mutation analysis in sputum or bronchoalveolar lavage fluid of patients with lung cancer. Takeda et al²¹⁴ reported the possible usefulness of mutant allele-specific amplification for detection of K-ras-positive cells in sputum of patients with lung cancer. In one of five sputum samples, a K-ras codon 12 alteration was found, whereas K-ras mutations in corresponding tumor specimens were not determined. Although this method is rapid as well, extreme efforts must be made to avoid contamination because a double amplification is required. The same is true for all enriched-amplification methods, which includes enriched single-strand conformational polymorphism described by Marchetti et al²¹⁵, who showed detection of the same p53 mutations in sputum that corresponded to tumor tissues in six patients with a primary tumor of the lung. Yakubovskaya et al¹⁰⁰ and Ronai et al²⁰² used enriched PCR, which included intermediate digestion to selectively eliminate normal alleles followed by polyacrylamide gel electrophoresis for visualization of the products. In that study, a high frequency of K-ras mutations was found in tumor tissues (63%) and sputum samples (49%) of patients with lung cancers. Furthermore, a relatively high frequency of K-ras alterations was found in control groups that included 10% of lung tissue from individuals with nononcological diseases, and even 12.5% of sputum samples of patients diagnosed with nononcological lung diseases carried a mutation for K-ras codon 12. This number of positive cases in the control group leads to an essential reduction in the specificity of this method and asks for specific controls during the experiment.

So far, two studies were reported on K-ras analysis of sputum specimens with known cytology data. As previously mentioned, Mao et al¹⁹⁶ used an allele-specific oligonucleotide hybridization technique for K-ras mutations in sputum specimens that corresponded to the tumor. Although the detection method used for this study has a high sensitivity and specificity and is able to detect rare lung cancer cells in cytologically negative sputum specimens, the method is time consuming and necessitates cloning and further allele-specific oligonucleotide hybridization using radioactive-labeled probes, which ultimately gives a result for the specific mutation in 2 to 3 weeks¹⁹⁶. Furthermore, Shiono et al²¹⁶ selected 12 sputum samples that were diagnosed as Papanicolaou class III or IV of 7,240 sputum samples for point mutation detection in K-ras codon 12. Using colony hybridization as the detection

method with a sensitivity of 1/1000 colonies, which is equivalent to the sensitivity of the former method, no K-ras codon 12 alterations could be shown in sputum samples. The relatively low frequency of 12 of 104 (12%) K-ras alterations found in tumor specimens of patients with adenocarcinoma of the lung shown by dot blotting can be explained by the low sample sensitivity of this detection method.

Using the PCR-PIREMA technique, Mills et al²⁰³ reported detection of K-ras mutations in bronchoalveolar lavage fluid for lung cancer diagnosis. Whereas the sample sensitivity of this method is extremely high, this method is time consuming and very cumbersome because it requires careful controls in two to three amplification and restriction enzyme steps. Using this protocol, an increased prevalence of K-ras mutations in lung adenocarcinoma was shown⁹⁹. The number of amplification cycles was lower in a PCR-based LCR approach, in which bronchoalveolar lavage fluids from five of 10 patients with lung cancer showed a mutation for K-ras codon 12¹⁹². However, for each LCR reaction, the use of radioactive-labeled primers was necessary, which, after denaturing gel electrophoresis, could be detected by exposure of the dried gel to film.

The Point-EXACCT method is rapid. Starting from genomic DNA from paraffin or frozen material, sputum specimens, or bronchoalveolar lavage fluids, point mutations can be detected within 10 hours. Therefore, Point-EXACCT requires considerably less time and effort compared with other techniques. Because Point-EXACCT uses only one amplification round, the risk of false-positivity possibly caused by numerous amplification cycles and PCR product handling is dramatically diminished.

The analysis of K-ras point mutations alone for early lung cancer diagnosis in sputum specimens may theoretically be useful for 15% of all patients with lung cancer, provided that tumor cells are present in the sample. Therefore, the investigation of more potential biomarkers for lung cancer, such as p53, is mandatory^{223,224}. Using a combination of markers, the sensitivity of mutation detection in lung cancer diagnosis may be increased. If molecular techniques will be used to prospectively screen sputum samples in people with a high risk for lung cancer, then, e.g., K-ras mutations may be detected, whereas on radiograph and computed tomographic-scan, no tumor is visible. Because (1) biologically, K-ras is found just before the visible occurrence of lung cancer in animal studies, and (2) in humans, it is a relatively late event in lung carcinogenesis, we believe that, when techniques with a high specificity are used, these people should be treated according to the guidelines for occult lung cancer of the American Thoracic Society and European Respiratory Society²²⁵. In addition, with the outcome of the present study, we believe that it is now worthwhile to initiate a large prospective study, in which K-ras is one of the parameters to be tested.

Other genetic alterations have been described in lung cancer that occur at a very early oncogenic stage, i.e., even in microscopically normal bronchial

epithelium of heavy smokers^{57,129}. Loss of heterozygosity at chromosome 3p, 5q, 9p and 17p was shown to have widespread occurrence in the bronchial epithelium^{57,129}. However, there appears to be no direct link between the occurrence of deletions at chromosome 3p in microscopically normal bronchial epithelium and the progression to malignancy, because these structurally acquired changes were present in greater than 80% of the smokers. As possible treatment, chemoprevention can be envisaged in this population to reduce the further derangement of genetic alterations.

In conclusion, with Point-EXACCT, K-ras point mutations were found in sputum specimens that were diagnosed negative for malignancy by cytological analysis. Therefore, molecular diagnostic analysis may be an important adjunct to cytology for the early diagnosis of lung cancer.

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Six

K-ras in non-malignant bronchial epithelium

Submitted as :

Do K-ras point mutations occur in non-malignant bronchial epithelium
of patients with lung cancer?

*Veerle AMC Somers, Arjen MJ van Henten, Guul PM ten Velde,
Barry Jutten, Solange H Korn, Jan-Willem Arends
and Frederik BJM Thunnissen*

Abstract

Point mutations in the Kirsten (K-ras) gene have been identified in non-small cell lung cancer (NSCLC) and specific K-ras mutational patterns have been observed after exposure to carcinogens. According to the field cancerization theory the entire epithelium of the upper and lower respiratory tract is at risk for development of invasive cancer as a result of exposure to carcinogens. To investigate this concept we studied the bronchial epithelium of patients with NSCLC. From 75 patients who were treated for NSCLC with curative surgery, K-ras point mutation analysis was performed on both tumor specimens and non-malignant bronchial brushes obtained at an area distant from the tumor location. Of the 75 tumors examined, point mutations of K-ras codon 12 were identified in 10% of squamous cell carcinomas, in 40% of adenocarcinomas and in 29% of large cell carcinomas. K-ras alterations were not identified in bronchial brushes obtained at an area distant from the tumor location. Our results prove that the presence of K-ras alterations is specifically related to tumor formation and further suggest that these changes do not occur on a widespread basis in the normal bronchial epithelium. These data lead to a refinement of the concept of field cancerization and suggest that a preferred susceptibility of certain cell types may exist for specific carcinogens.

1. Introduction

It is generally accepted that carcinogenesis of the lung is a multistep process including several morphologic and genetic changes. The sequential events of these changes are commonly termed preneoplastic changes. For squamous cell carcinomas, i.e. tumors arising in the central part (originating in bronchi) of the respiratory epithelium, a well accepted progression of morphologic changes has been defined. These changes include hyperplasia, squamous metaplasia, dysplasia and carcinoma in situ^{147,222}. For the definition of basal cell hyperplasia it is essential to realize that parabasal cells occur in the larger airways of normal lungs¹²⁶. Adenocarcinomas mainly arise in the periphery of the lung and are thought to originate from cylindrical epithelium in the bronchioli, e.g. Clara cells and alveolar type II pneumocytes²²⁶. Hyperplastic and dysplastic changes associated with these peripheral tumors are not well documented. Although the presence of atypical alveolar hyperplasia (AAH) has been suggested as a possible premalignant precursor lesion of adenocarcinomas²²⁷⁻²²⁹, the significance of AAH in the pathogenesis of lung adenocarcinoma is still a matter of controversy^{230,231}.

Currently, it is not clear to what extent premalignant changes in lung tumorigenesis involve the respiratory epithelium at distant areas from the

tumor origin. Smith and coworkers demonstrated DNA aneuploidy in focal areas of preneoplasia or carcinoma in situ in patients with aneuploid NSCLC¹⁴⁴. This finding supports the results of earlier quantitative cytochemical studies, demonstrating the presence of increasing amounts of DNA in exfoliated cells from abnormal epithelia^{232,233}. The presence of aneuploidy denotes crude quantitative changes ($>1\text{-}2\%$) in total amount of DNA. In addition, this finding in multiple preneoplastic foci located throughout the respiratory epithelium supports the field cancerization theory.

Initially, the phenomenon of field cancerization was reported as the occurrence of multiple carcinomas arising in patients with oral squamous cell carcinoma. This may be caused by the massive exposure of the upper and lower respiratory tract epithelium to carcinogens present in tobacco smoke, which ultimately results in the independent initiation of multiple epithelial cells¹⁵². In addition, patients with a laryngeal carcinoma have a high risk for a metachronous second primary lung carcinoma^{152,234}. Both primary carcinomas may occur synchronously with squamous metaplasia of the lower respiratory tract¹³².

While the phenomenon of field cancerization is based on clinical observations, the biologic and genetic changes responsible for this process are not fully understood. However, support for the field cancerization theory came from Sozzi and coworkers, who identified different genotypes with regard to p53 mutations and chromosome 3 status in metachronously arising tumors²³⁵. Recently, a novel mechanism for field cancerization was described by Franklin and coworkers, who showed the widespread presence of a single somatic p53 mutation in the bronchi of a smoker, suggesting that a single progenitor bronchial epithelial cell clone may expand to populate broad areas of the bronchial mucosa²³⁶.

So far, a number of genetic changes have been reported in preinvasive lesions of the lung. Loss of heterozygosity (LOH) for chromosome 3p, 9p, 17p and p53 alterations have been found in dysplastic tissue in proximity to lung cancer resection specimens and in premalignant lesions of the lung^{63,128,130,131}. In recent studies, of morphologically 'normal' bronchial epithelium of smokers widespread LOH on chromosome 3p, 5q, 9p and 17p was found^{57,129}. Taken together, these data suggest independent genetic origins of cancer within the epithelial field at risk.

Regarding a possible role of K-ras alterations in the process of field cancerization in the lung, no consensus exists. Point mutations in the K-ras gene have been identified in 30% of adenocarcinoma subtypes using point mutation detection methods with relatively low sample sensitivity^{78,80,81,97,112}, whereas the presence of these alterations increased to 50% in adenocarcinoma subtypes and 19% of squamous cell carcinomas using highly sensitive methods^{99,104,199,217}. Although the role of K-ras alterations in the carcinogenesis cascade has been subject of many studies, it is not yet clear if these alterations occur in distant normal, metaplastic, or dysplastic lung

specimens from patients with NSCLC. Several studies used microdissection to specifically analyze normal or preneoplastic cells^{92,108,109,148} adjacent or distant from the tumor location for the presence of K-ras alterations^{204,237}. In some studies the occurrence of K-ras point mutations has only been shown in the tumor but not to normal lung tissue²³⁸. However, another study described the identification of these alterations in malignant tumor and adjacent normal tissues, but not in normal appearing cells at greater distance from the tumor²³⁷. Moreover, other groups report K-ras point mutations even in non-malignant tumor tissue collected at greater distance from the tumor^{100,239,240}. In general, the magnitude of the distance from the tumor was not specified. Thus, the issue of the presence of K-ras codon 12 alterations is still controversial.

K-ras point mutations have been demonstrated in sputum samples from patients with adenocarcinoma of the lung. Since sputum samples usually contain a limited number of bronchial epithelial cells, these cells might lead to a positive result if they harbor a K-ras mutation. Thus, for carcinogenetic interest and for the possible use of K-ras as biomarker in lung cancer screening further elucidation of the relationship between K-ras and non-malignant human bronchial epithelium is mandatory.

The aim of this study was to examine to what extent K-ras point mutations are present in human bronchial epithelium of patients with NSCLC. To this end, 10^6 - 10^7 bronchial epithelial cells were collected with brushes and investigated with Point-EXACCT (point mutation detection using exonuclease amplification coupled capture technique), which has a high sample sensitivity^{197,217,241}.

2. Materials and Methods

2.1 Patients enrollment

The study involved 75 randomly selected patients with NSCLC who underwent lobectomy or pneumectomy at the Maastricht University Hospital, Maastricht between November, 1992 and August 1997. Patient characteristics relevant for this study were selected from patient files. Some of the resection specimens were used in a previous study²¹⁷.

2.2 Tumor specimens

After pneumectomy or lobectomy of the tumor, lung specimens were transported within 10 minutes to the pathology department for clinical evaluation of the resection margins. Fresh tumor tissue was stored at -70°C immediately after resection. Concomitantly, tumor tissue was fixed in formalin

and embedded in paraffin for histopathologic classification on 5 μ m hematoxylin and eosin stained sections. Histopathologic examination of the tumors was performed according to the World Health Organization guidelines²⁰⁰. Genomic DNA of tumor specimens was isolated from 25- μ m frozen sections, using a rapid lysis procedure¹⁹⁷. The concentration of DNA was estimated on a 1% agarose plate with a known DNA-standard for comparison.

2.3 Bronchial brushes

For acquisition of bronchial brush specimens in 73 cases, a proximal bronchial ramification was selected which was not leading to the tumor area. Bronchial epithelial cells were collected from second to fourth order bronchi. Since in the remaining two cases the tumor was obstructing the largest bronchus including ramification, the bronchial epithelium proximal to the tumor was collected. A free margin of at least 0.5 cm was kept to the tumor during brushing. Ten bronchial brushes were obtained from each patient for DNA isolation and one additional bronchial brush was used for cytologic investigation. With each brush approximately 0.5×10^6 non-malignant human bronchial epithelial cells were obtained.

For the first 33 patients, bronchial brush material was sampled in 4 ml of culture medium (Dulbecco's modified Eagle's medium; DMEM) to which 4 ml 8M guanidium isothiocyanate was added, as described elsewhere²⁴². This method allowed both RNA and DNA isolation after ultracentrifugation of the specimens. After ultracentrifugation, total RNA was present in the pellet and DNA was further isolated by standard phenol-chloroform extraction. DNA concentration and purity was determined by A260/A280 measurements and checked with 1% agarose gel electrophoresis. DNA was used for amplification and K-ras point mutation analysis in this study.

For the remaining 42 patients, the bronchial brush material was collected in 5 ml 1 x phosphate-buffered saline (PBS) and kept on ice. Bronchial epithelial brushes in 5 ml PBS were centrifuged for 15 minutes at $413 \times g$ at room temperature. After centrifugation, the supernatant was discarded and the remaining cell pellet was stored at -20°C . For DNA isolation of these bronchial brush specimens, excess blood was first eliminated by lysis of the specimens for 5-10 minutes at room temperature in 1 x lysis buffer (155 mM NH_4Cl /10 mM KHCO_3 /0.1 mM EDTA). After centrifugation for 5 minutes at $413 \times g$, the supernatants were discarded and the remaining cell pellets were washed twice with 5 ml 1 x PBS. Genomic DNA was isolated from cell pellets using a rapid lysis procedure, as described elsewhere¹⁹⁷.

Cytologic examination in the brush specimens was performed on Giemsa stained smears. All cytologic findings were reviewed, and the presence of

metaplasia with or without atypia or tumor cells was recorded. In addition, an estimate was made about the cellular composition of the brush specimens.

2.4 K-ras exon 1 gene amplification and point mutation analysis by Point-EXACCT

A 204 bp product of the first exon of the K-ras gene was amplified using primers KPR3 and KI8 under conditions previously described¹⁹⁷. One microgram of tumor and bronchial brush DNA was used for amplification by polymerase chain reaction (PCR) for 40 cycles in a total reaction mixture of 100 μ l.

Point mutation analysis was performed according to the conditions described before in detail, with probes directed against base 1 and 2 of K-ras codon 12¹⁹⁷. Probe conditions and corresponding ligase conditions for base 2 of K-ras codon 12 were slightly modified as described in recent reports^{217,241}.

2.5 Control specimens

Amplification and point mutation detection by Point-EXACCT included in each experiment multiple positive and negative controls^{197,217,241}. Amplification products containing a known mutant fraction of 1/15,000 wildtype cells were used in each experiment as external positive control for the sensitivity. Furthermore, all samples were separately tested with each of 4 different base specific probes (G, A, T and C). At least one of these A, T or C probes functions as an internal negative control. Therefore, positivity levels in the Point-EXACCT assay for K-ras codon 12, base 1 and base 2 separately were determined on both external and internal controls as previously described²¹⁷.

3. Results

3.1 Patients

The study population included 60 men and 15 women. The age distribution ranged from 36 to 81 (mean age 65 years). There were 47 patients with squamous cell carcinomas, 21 with adenocarcinomas and 7 with large cell carcinomas. According to the tumor-node-metastasis (TNM) classification system, 40 patients were stage I, 24 were stage II, 5 were stage IIIA, 4 were stage IIIB and 2 were stage IV. From 66 out of 75 patients, smoking status could be traced. All patients except one were smokers, of which 30 patients were current smokers and 35 patients were ex-smokers. From the smoking population mean pack-years of tobacco use was 40 ± 21

(range <1 to 106), and for smoking duration these values were 41 ± 14 years (range <1 to 62).

3.2 K-ras codon 12 point mutation analysis

In 15 out of 75 (20%) tumor samples a K-ras codon 12 mutation was identified. Clinical characteristics of patients with a K-ras mutation in tumor specimens are represented in table 1. K-ras point mutation analysis was positive for a mutation in 10% of squamous cell carcinomas, in 40% of adenocarcinomas and in 29% of large cell carcinomas. K-ras alterations identified in tumor specimens consisted of 6 G-A transitions, resulting in 4 serine mutations and 2 aspartic acid mutations, and 8 G-T transversions were found, coding for a cysteine mutation in 2 cases and a valine mutation in 6 cases. One G-C transversion was demonstrated resulting in an alanine mutation. The majority (67%) of mutations was found in patients with stage I disease.

We further investigated the relationship between tobacco use and the presence of K-ras alterations. Of the 75 patients analyzed, the 9 patients without a known smoking status were negative for a K-ras alteration. Of the remaining 66 patients, mean pack-years of tobacco use did not differ between patients with K-ras positive tumors (37 ± 15) and patients with K-ras negative tumors (41 ± 23). Based on duration of smoking these values were 38 ± 15 for K-ras positive tumors, and 42 ± 14 for K-ras negative tumors. In addition, the percentage of patients with K-ras positive tumors was not influenced by the number of pack-years or smoking duration. No difference in K-ras positivity was found between patients with current smoking status and ex-smokers (23.3% versus 22.8%). Noteworthy is that K-ras point mutations were identified in 5 patients who quit smoking several years (6, 6, 16, 20 and 20, respectively) before resection of the tumor.

Table 1. Clinical characteristics of patients containing a K-ras mutation in tumor specimen

No.	Age/G	Tumor type	T-N	Tobacco use ^a	Pack-years	K-ras ^b
3	71/M	squamous	2-0	ex (years)	45	GTT
14	68/M	squamous	1-0	ex (years)	25	GTT
19	73/M	squamous	2-0	current	60	AGT
32	52/F	adenoca	2-0	current	27	GTT
34	47/M	large cell	1-0	current	45	AGT
38	58/F	adenoca	1-0	current	42	TGT
47	68/M	adenoca	2-0	ex (months)	54	GAT
54	68/F	adenoca	2-1	no	<1	GTT
65	67/M	large cell	4-2	current	45	TGT
68	58/M	squamous	3-0	current	38	AGT
70	71/M	adenoca	1-1	ex (>20 years)	14	GTT
71	69/F	adenoca	1-0	current	46	GAT
72	71/F	adenoca	2-2	—	40	GTT
73	73/M	squamous	1-0	ex (years)	50	AGT
75	71/M	adenoca	1-0	ex (years)	26	CGT

G: gender; T: tumor; N: normal; M: male; F: female; squamous = squamous cell carcinoma, adenoca = adenocarcinoma, large cell = large cell carcinoma, — = unknown

^ainformation in parentheses indicate time since last cigarette

^bThe normal codon 12 sequence is GGT, coding for glycine

3.3 Bronchial brushes

The collection of bronchial epithelial cells by brushing of second to fourth order bronchi at an area distant from the tumor location resulted in a large number of cells useful for molecular analysis. Cytologic examination of Giemsa stained slides was performed on 75 cases. In most slides alveolar macrophages occasionally related to a small mucus strand were discerned. The mean and standard deviation of the percentage of alveolar macrophages was $3.4\% \pm 4.5\%$ (range 0-15). In 4 cases signs of acute inflammation were present with a range of 2-15% of neutrophilic granulocytes (mean $8\% \pm 5\%$). The majority of the cells were bronchial epithelial cells ($96.2\% \pm 5.7\%$). In

18 bronchial brushes metaplastic cells were present with atypia in 5 cases. Four specimens showed a limited number of tumor cells. In one of these 4 cases the bronchial epithelium proximal to the tumor was collected.

An average DNA content of $71 \mu\text{g} \pm 51$ (range 16.2 to 130.5) was obtained from the bronchial brush specimens. All bronchial brush specimens from the group of 75 patients could be successfully amplified resulting in dense amplification products. In addition, point mutation analysis by Point-EXACCT gave reliable optical density signals for the G probe (internal positive control) which were comparable to control specimens.

The Point-EXACCT analysis showed no K-ras alterations in bronchial cells from the 15 patients with a K-ras positive tumor. Similarly, in none of the bronchial brush specimens from the 60 patients with a K-ras negative tumor a point mutation for K-ras codon 12 was demonstrated. Importantly, our external control samples with mutant fraction 1/15,000 were found positive in all experiments and the internal positive control was positive in each case (data not shown). The four cases with tumor cells in the bronchial brush specimen were from patients with K-ras negative tumor. In 4 from the 15 patients with a K-ras positive tumor metaplasia was discerned in the bronchial brush.

4. Discussion

The present study clearly demonstrates that K-ras codon 12 alterations do not occur in non-malignant bronchial epithelial cells, not even in patients with K-ras positive tumors.

The Point-EXACCT method has a high sample sensitivity, i.e. one malignant cell with a mutation can be discerned between 12,000-15,000 genetically normal cells. This number of cells is generally investigated for each nucleotide with Point-EXACCT. Because the positive control (1 cell with a K-ras alteration in 15,000 genetically normal cells) was detected in each experiment it is very unlikely that even a single bronchial epithelial cell with a K-ras alteration would be missed.

The distance between bronchial epithelial cells collected with brushing and the tumor may vary from 0.5-5 cm. At this distance from the tumor the occurrence of K-ras alterations was not perceived. This finding is in agreement with the results of a previous study of Santos and coworkers who analyzed both tumor and normal tissue of one patient with a squamous cell carcinoma for the presence of a K-ras alteration²³⁸. Contradictory results have been obtained by Clements and coworkers, who analyzed endobronchial biopsies from patients with smoking histories undergoing diagnostic bronchoscopic evaluation for the presence of K-ras alterations²³⁹. In that study, K-ras alterations were found in distant non-malignant tissue from five patients with

a pulmonary malignancy as well as in two patients without a malignancy. Moreover, the K-ras alteration was not necessarily identical in both tumor and non-tumor tissue²³⁹. These results were also based on a relatively low number of paired tumor and non-tumoral tissue specimens. This study has two remarkable findings. Firstly, with single-strand conformation polymorphism (SSCP) a relatively high percentage (50%) of K-ras alterations was detected in adenocarcinomas. Based on the sample sensitivity of this technique, usually up to 30% of K-ras alterations were found in adenocarcinomas of the lung^{96,123,243}. In addition, a high percentage of G-C transversions was identified, an uncommon observation made in patients with NSCLC. Although in the present study more patient samples have been investigated combined with a higher number of cells studied in each patient, we were not able to confirm their results. In another study Clements and coworkers reported a 32% mutation frequency of K-ras alterations in lung carcinomas with PCR-SSCP analysis, of which the mutational spectrum revealed a predominance of G-T transversions²⁴⁰. Interestingly, K-ras alterations were also identified in 29% of non-malignant peripheral lung tissue samples in patients with lung cancer. Whether this phenomenon is due to the presence of AAH is not known since the morphology of these specimens was not mentioned. This may be a possible explanation for their results, which are in this case not at variance with our findings, since K-ras alterations have been demonstrated in AAH¹⁰⁸.

With microdissection techniques the number of cells studied can range from 50-500 cells^{92,108,109}. Due to this relatively low number of cells investigated with these techniques a chance exists for underestimation of positive cases. In the present study a 75 fold number of cells has been investigated. Thus, the fact that no K-ras alterations were identified in normal bronchial epithelial cells reduces the chance of 'false' negativity.

The high number of epithelial cells obtained from the brush specimens also allowed cytologic examination, which showed metaplastic changes in 25% of the specimens examined, occasionally accompanied with atypia. We did not identify K-ras alterations in any of these specimens. This observation is in agreement with a previous study denoting no direct histologic transition between peripheral tumors and more dysplastic and metaplastic lesions in the larger airways⁹².

In recent studies, LOH has been demonstrated to occur widespread in the morphologically 'normal' bronchial epithelium of >80% of smokers^{57,129}. From the majority of the patients in the present study is known that they were heavy smokers based on the number of pack-years and smoking duration. Although the presence of LOH is likely in the DNA of these patients, the absence of K-ras alterations excludes a possible role of these alterations in the process of carcinogenesis in the epithelial cells of the larger airways. In these airways the cellular composition consists of ciliated cells, goblet cells, neuroendocrine cells, basal cells and parabasal cells. The major differences in epithelial cells between the central and peripheral airways is the presence of

Clara cells in the smaller bronchioli and type II pneumocytes along the alveoli. Assuming that specific metabolic processes occur before a certain genetic alteration arises, and the absence of K-ras alterations in the larger airways, makes it unlikely that the cell types in these airways are capable of the 'K-ras related' metabolism. Interestingly, the Clara cell and the alveolar located type II pneumocytes may differ in their xenobiotic metabolic activity from other bronchial epithelial cells^{244,245}. Therefore, this study provides arguments that at least one of these two cell types may be a stem cell for adenocarcinoma of the lung. In addition, this may refine the field cancerization concept with preferred susceptibility of certain cell types above others to specific carcinogens.

Our study was not designed to investigate a relationship between the presence of K-ras alterations and smoking. No differences in the frequency of mutations were observed between current smokers and patients with a smoking history. However, the identification of K-ras alterations in patients who stopped smoking several years before resection of the tumor, even more than 20 years, may indicate that either K-ras alterations can arise due to exposure to other carcinogenic substances than smoking or that, if K-ras alterations are due to smoke exposure, these should be present in precursor cells for more than 20 years without giving rise to a tumor.

K-ras point mutations have been demonstrated in sputum samples from patients with adenocarcinoma of the lung^{196,241}. Since sputum samples usually contain a limited number of bronchial epithelial cells, these cells might lead to a positive result, if they harbor a K-ras mutation. However, the results of this study show no K-ras positivity in bronchial epithelial cells, thereby excluding the theoretical possibility of 'false' positivity in the possible use of K-ras as biomarker in lung cancer screening.

In conclusion, with Point-EXACCT K-ras point mutations were found in tumor specimens of patients with NSCLC but not in non-malignant bronchial epithelial cells collected at distant areas from the tumor location. This proves that the presence of K-ras alterations is specifically related to tumor formation and further suggests that these changes do not occur widespread in the bronchial epithelium

Seven

K-ras in bronchial wash fluids

In press as :

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

*Veerle AMC Somers, Arjen MJ van Henten, Guul PM ten Velde,
Jan-Willem Arends and Frederik BJM Thunnissen*

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Abstract

To examine the additional diagnostic value of K-ras point mutations in the clinical diagnosis of peripheral lung tumors, bronchial wash fluids obtained during bronchoscopy of patients suspected for lung cancer were studied. Only those patients were investigated of which the cytologic diagnosis was not conclusive for malignancy. As a control group patients without lung cancer were investigated. Point mutation analysis of K-ras codon 12 with Point-EXACCT was performed in bronchial wash fluids and corresponding tumor tissues, if available. With Point-EXACCT K-ras alterations were identified in 4 out of 19 (21%) bronchial wash fluids without a decisive diagnosis of malignancy. Diagnosis of malignancy was based on cytologic examination of bronchial brush specimens of 7 patients, a transthoracic needle aspiration of two patients, a histologic investigation of biopsy specimens of 5 patients (including one patient with a positive bronchial brush specimen), resection specimens of 3 patients, a needle aspiration of a lymph node in the neck of one patient and a pleura fluid of the last patient. Analysis of bronchial wash fluids of 11 patients without lung cancer revealed no K-ras codon 12 alterations. In conclusion, the identification of K-ras alterations in bronchial wash fluids obtained during bronchoscopic procedure denotes the use of K-ras as a biomarker in clinical diagnosis of lung cancer and may serve as an adjunct to cytology in lung cancer diagnosis.

1. Introduction

Lung cancer is a common and deadly disease. At the time of diagnosis, lung cancer is often locally or systemically advanced. Therefore, only about 25% of all patients with lung cancer present with locally defined tumors which are considered amenable to surgical resection²⁰.

Patients being evaluated for suspected lung cancer based on the presence of a lung mass on an X-ray of the thorax often receive a diagnostic fiberoptic bronchoscopy during which a biopsy is taken for tissue diagnosis and additional bronchial brushes and wash fluids are performed for cytologic examination. It is generally accepted that in the absence of a histologic diagnosis treatment for lung cancer can be based on cytologic diagnosis of malignancy in bronchial brush or wash specimen. In case of a lung tumor, which is inaccessible for biopsy, cytologic examination of the wash specimen may be the only diagnostic modality. However, the cytologic diagnosis is sometimes equivocal, especially in specimens where only a limited number of tumor 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 fairly characteristic for lung cancer. 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^{80,99,104,112,217,246}. These alterations have also been identified in bronchoalveolar lavage fluids of patients with suspected lung cancer^{192,203,247}.

In a recent study, we have successfully applied a highly sensitive method Point-EXACCT for K-ras point mutation detection in sputum of patients with adenocarcinoma of the lung²⁴¹. 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 whether this technique can be helpful in determining the nature of a peripheral lung tumor. The identification of these alterations in clinical specimens, which are not diagnostic for malignancy, may improve the diagnostic yield of the fiberoptic bronchoscopic procedure.

The aim of this study was to examine the additional diagnostic value of K-ras point mutations in bronchial wash fluids of patients under suspicion of lung cancer. To this end, a consecutive series of bronchial wash fluids without malignant cytologic diagnosis was investigated with Point-EXACCT in patients with abnormalities on X-ray and in a control group.

2. Materials and Methods

2.1 Patients

From January-May 1997, 140 patients underwent diagnostic fiberoptic bronchoscopy at the University Hospital Maastricht. Indication for bronchoscopy was that a lung mass was visible on X-ray or the persistence of chronic cough. The routine diagnostic procedure consists of bronchial brushing and washing for cytologic investigation and bronchial biopsy for histologic examination. The cytologic diagnosis can be categorized into 4 groups: 1) no malignant cells present; 2) atypia, when the cytologist notices slight changes, but expects these still to be benign; 3) suspicious for malignancy where the cytologist finds abnormal cells, but a definite diagnosis of malignancy cannot be made. This may be due to only a very limited number of tumor cells, and/or minimal deviation from the normal range; and 4) malignant cells, where the cytologic diagnosis is certain, keeping the desired 100% specificity for malignancy in mind. In 19 patients with a peripheral lung lesion the cytologic diagnosis of malignancy was not made in bronchial wash specimens. These were examined for further molecular study. In addition, bronchial wash fluids

from 11 patients with non-oncologic diseases were used as controls. The molecular examination was performed without knowing the status of the clinical diagnostic procedure.

2.1 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 tumor specimens were obtained when available and further used for isolation of DNA. Biopsy and resection specimens were available from 16 patients. Paraffin sections (5 x 20 m) were dewaxed in xylene and rehydrated. DNA was isolated using proteinase K digestion¹⁹⁷. Of sixteen biopsy or resection specimens collected, 7 were used for DNA isolation and molecular analysis.

2.2 Cytology

Bronchial wash fluids were performed according to a standardized protocol²⁴⁸. In brief, after local anaesthesia of the upper respiratory tract, the fiberoptic bronchoscope was securely wedged in an airway adjacent to the radiographically defined lesion. The wash fluid was directly mixed in an excess of alcohol-carbowax and subsequently transported to the pathology department for further processing of the specimens. Specimens were first put in the blender to break up eventual mucus. After centrifugation of the specimens at 560 x g for 10 minutes, the pellets were used for preparation of 2 smears. These were alcohol fixed and Papanicolaou stained. The remaining of the specimens was further centrifuged at 413 x g for 7 minutes and the pellets were washed 3 times in 4 ml alcohol 96%. Specimens were mixed gently and further centrifuged at 413 x g for 7 minutes. The supernatants were discarded and the pellets were dissolved in 50-400 μ l lysis buffer for DNA isolation, depending on the number of cells¹⁹⁷.

2.3 K-ras codon 12 point mutation analysis

Amplification of the first exon of the K-ras gene was performed with primers of the first exon of the K-ras gene outside the codon 12 region¹⁹⁷. The Point-EXACCT method for point mutation analysis in K-ras codon 12 was performed as described¹⁹⁷, with probe and corresponding ligase conditions for base 1 and 2 as described^{217,241}.

All samples were subjected twice to both amplification and point mutation analysis by Point-EXACCT. Samples with signals above the controls in both experiments are called positive. Each amplification and point mutation detection experiment included several positive and negative controls.

3. Results

The Point-EXACCT method was used to identify K-ras point mutations present in bronchial epithelial cells from bronchial wash fluids of 19 patients, 16 men and 3 women (age range, 56 to 82 years) under suspicion of lung cancer. All patients presented with a peripheral lung lesion on the X-ray of the thorax, but in 10 patients 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 patient had a lesion in the neck. The majority of lesions (11 of 19 lesions, 58%) were located in the right upper lung.

K-ras codon 12 alterations were found in 4 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 4 bronchial wash specimens positive for a K-ras alteration were found in patients without a decisive cytologic diagnosis of malignancy. Only two of the 4 patients with a K-ras positive bronchial wash specimens were diagnosed 'suspicious for malignancy' with cytologic examination, whereas no malignant cells were found in the remaining two specimens positive for a K-ras alteration. The earliest detection of K-ras positive cells in bronchial wash fluids was obtained 38 days before final diagnosis of malignancy.

Diagnosis of malignancy was in the group of 19 patients based on cytologic examination of bronchial brush specimens of 7 patients, a perthoracic needle aspiration of two patients, biopsy specimens of 5 patients (including one patient with a positive bronchial brush specimen), resection specimens of 3 patients, a needle aspiration of a lymph node in the neck of one patient and a pleura fluid of the last patient. In only 7 patients, diagnosis of malignancy was made during the first diagnostic bronchoscopic procedure, whereas in the remaining 12 patients, further invasive diagnostic procedures were necessary, which in some cases yielded a diagnosis of malignancy after several months (table 1). Among the 19 patients with radiographically suspected and histologically or cytologically confirmed carcinomas, the most frequently observed subtype was adenocarcinoma (8 cases).

Of the 4 patients with a K-ras positive bronchial wash specimen, in two an endobronchial tumor was seen. In one patient 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 further made on a pleura fluid aspiration. In the remaining 2 patients without a visible endobronchial tumor, the brush revealed malignant cells in one patient, whereas in the other patient the clinical diagnosis of malignancy was histologically confirmed after lobectomy.

In 7 of 19 patients with lung cancer, histologic tumor specimens were examined for the presence of K-ras alterations with Point-EXACCT. Three of the 7 cases showed the wild type sequence GGT for K-ras codon 12. In two of the 4 K-ras positive tumors the corresponding wash specimens showed the same K-ras alteration. In the remaining 2 patients with a K-ras positive tumor the bronchial wash fluid was negative, which renders the presence of tumor cells unlikely. Interestingly, no K-ras alterations were found in bronchial wash fluids of K-ras negative tumors.

The histologic classification of the K-ras positive tumor specimens showed 3 adenocarcinomas and one large cell carcinoma. The mutations identified in biopsy or tumor specimens from surgical resection were 2 G-A transitions, which resulted in a serine mutation, whereas one G-T and one G-C transversion were detected which coded for a cysteine and an alanine mutation, respectively.

Table 1. Clinical characteristics of patients with corresponding tumor and bronchial wash fluids

Age/G	Tumor Site	Cytol. ^a wash	K-ras ^b wash	Final diagnosis carcinoma ^c	Procedure to diagnosis ^d	Time to diagnosis ^e	K-ras ^b tumor
1. 71/M	RU	1	GGT	Adeno	biopsy (3)	143	CGT
2. 82/M	LU	1	GGT	Adeno	biopsy (3)	44	-
3. 64/M	lingula	1	GGT	SCLC	brush (1)	-	-
4. 81/M	RU	3	GGT	SqCC	biopsy (1)	-	-
5. 71/F	RM	1	GGT	Adeno	per. needle (2)	19	-
6. 63/F	RU	1	GGT	Adeno	per. needle (2)	7	-
7. 56/M	neck	1	GGT	Meta larynx	needle (2)	13	-
8. 66/M	RU	1	GGT	LCC	resection (3)	42	GGT
9. 77/F	LU	3	GGT	SCLC	brush (1)	-	-
10. 70/M	RU	2/3	AGT	Adeno	resection (2)	37	AGT
11. 58/M	RU	2	TGT	LCC	brush (1)	-	TGT
12. 64/M	lingula	1	GGT	LCC	brush (2)	27	-
13. 78/M	RU	1	AGT	Adeno	pleura fluid (3)	38	-
14. 71/M	RU	3	TGT	Adeno	brush (1)	-	-
15. 66/M	RU	1	GGT	SqCC	brush/biopsy (1)	-	GGT
16. 59/M	RU	1	GGT	Adeno	resection (2)	22	AGT
17. 62/M	RB	2	GGT	LCC	biopsy (2)	12	GGT
18. 68/M	LU	3	GGT	SCLC	brush (1)	-	-
19. 73/M	RU	1	GGT	LCC	biopsy (4)	34	-

G: gender; R: right lobe; L: left lobe; U: upper lobe; M: middle lobe; L: lower lobe; B: bronchus; per. needle: perthoracic needle

^a 1: benign; 2: atypia (probably benign); 3: suspicious for malignancy; 4: malignant

^b The normal codon 12 sequence is GGT, coding for glycine

^c SqCC, squamous cell carcinoma; Adeno: adenocarcinoma; LCC: large cell carcinoma; SCLC, small cell lung carcinoma; Meta: metastasis

^d number of diagnostic procedures leading to diagnosis of malignancy

^e time in days between bronchial washing and diagnosis of malignancy

As a control group, we further analyzed bronchial wash specimens obtained from 11 patients with either chronic bronchitis (1), COPD (1), abscess (2), pneumonia (3), persistent infiltrate (2), interstitial infiltrate (1), and a scopy in a patient with a tonsil carcinoma (1). These patients did not present with a tumor lesion later in time (14-18 months follow-up). Of the total of 11 cases analyzed, 8 were men and 3 were women, with age ranging from 46 to 74 years. By routine cytologic investigation all 11 cases were classified into category 1, which coded for no malignant cells present. Point mutation analysis on K-ras codon 12 by Point-EXACCT did not reveal any mutation.

4. Discussion

Patients with a peripheral lung mass on radiograph under suspicion of lung carcinoma undergo bronchoscopic procedure, including bronchial biopsy and brush specimens. The inaccessibility of the bronchoscope to the affected peripheral region frequently fails to yield a status of malignancy, and, as a consequence, diagnosis of malignancy is made by cytologic examination of the bronchial wash fluid. Beside a possibly better sampling of the peripheral tumor cells in the bronchial wash specimens, the diagnosis of malignancy is sometimes equivocal, especially in cases in which only a very limited number of tumor cells are present in the clinical specimen. From those patients of whom the clinical consequences of a K-ras positive bronchial wash specimens were later ascertained, the Point-EXACCT method could offer clinical diagnostic potential for malignancy.

With the use of Point-EXACCT, K-ras alterations were identified in bronchial wash specimens of 4 out of 19 patients under suspicion of lung cancer, whereas cytologic examination was not diagnostic for malignancy. In these 4 patients a molecular diagnostic support for the diagnosis of malignancy is present. This leads to an earlier diagnosis of malignancy and avoids the need for additional invasive diagnostic procedures.

At present, the question raises whether diagnosis of malignancy can be based on the identification of a K-ras alteration in bronchial wash specimens

without cytologic prove of malignancy. In consideration of the present study a number of issues arise with the use of this molecular diagnostic approach for early diagnosis of malignancy.

With routine cytologic investigation only part of the cells, obtained by bronchial washing are studied, i.e. in this procedure, diagnosis of malignancy is based on examination of a limited number of cells which are present in smears on 2 slides. A minimal number of tumor cells and/or minimal deviation from normal may explain false negative results of the cytologic diagnostic procedure. In contrast, the particularly high number of cells studied with Point-EXACCT, e.g. about 12,000-15,000 cells per nucleotide, makes the finding of K-ras positive cells very likely. The alternative explanation for a 'false negative' diagnostic test is the absence of tumor 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 about 50% of the adenocarcinomas, which represent the majority of peripheral lung carcinomas. Squamous cell carcinomas and small cell lung carcinomas (SCLC) are usually centrally located. For these tumor types, the additional value of K-ras gene analysis is limited in our study. This observation is consistent with the results of other studies, which indicate no K-ras alterations in SCLC subtypes and only in a minority of squamous cell carcinomas^{93,249,250}. However, the centrally located tumors have a higher chance to be visible by bronchoscopy and to be diagnosed by brush and/or biopsy. Importantly, by investigating bronchial wash fluids for p53 alterations, e.g. in patients suspected for a small cell lung carcinoma (SCLC) the sensitivity of detection could be considerably improved^{74,77}.

By determining K-ras in bronchial wash specimen for diagnostic purpose the question arises, if a positive test, i.e. the presence of K-ras point mutation, is always associated with lung cancer. In case of peripheral lesion on the X-ray of a patient >40 years of age, the chance for a lung carcinoma is high, especially if a smoking history is present. The chance of a benign lesion on the X-ray is estimated at 40%. In theory, a situation may exist that the Point-EXACCT method is positive for a K-ras alteration and that the lesion is benign: e.g. a hamartoma with adjacent K-ras positive atypical alveolar hyperplasia (AAH). An approximation of the chance of this occurrence is obtained by multiplication of the individual chances (benign lesion, 40%^{251,252}) x (incidence AAH in resection specimens of all cancers, 5%²⁵³) x (K-ras positivity in AAH, 25%⁹²) equals 0.5%. This chance is similar to the false positive rate of cytology. Therefore, we feel comfortable with the decision of curative surgery based on a shadow on the X-ray plus K-ras positive Point-EXACCT test in case of non-conclusive routine diagnostic procedure.

Nowadays, highly sensitive molecular techniques are used for detection of these molecular alterations in several excretions/secretions. These specimens often contain the same genetic alteration corresponding to the

tumor. The presence of genetic alterations has been demonstrated in several body fluids such as effluent samples of patients who have undergone colonoscopy, yet were free of colorectal diseases at the time of examination²⁵⁴, faecal material of patients with colorectal cancer^{100,210}, pancreatic juice of patients with pancreatic cancer^{212,255} and urine obtained at surgery of patients with bladder cancer²¹¹.

In previous reports, the use of K-ras alterations in bronchoalveolar lavage fluids has been described as a clinically useful marker for lung cancer diagnosis^{192,203,247}. In this study, K-ras codon 12 alterations were only found in bronchial wash fluids of patients suspicious for lung cancer but not in a series of patients without oncologic disease. These data denote the high specificity of the Point-EXACCT method and further affirm the use of K-ras alteration as a marker in the clinical diagnosis of peripheral lung tumors. Our data are consistent with the results of Mills and coworkers who did not find any K-ras alteration in specimens with diagnosis other than NSCLC²⁰³. Contradictory results have been obtained in a study by Yakubovskaya and coworkers, who found a 12.5% frequency of K-ras alterations in patients with non-oncologic disease¹⁰⁰. In that study, the lower specificity could be improved by inclusion of specific controls during point mutation analysis.

In conclusion, the present study demonstrates that the presence of K-ras alteration in bronchial wash fluids is clinically useful in diagnosis of peripheral tumors and that molecular analysis can serve as an important adjunct to bronchoscopy in lung cancer diagnosis.

Eight

K-ras and prognosis

Submitted as :

The prognostic value of specific K-ras point mutations
in non-small cell lung carcinomas

*Veerle AMC Somers, Guul PM ten Velde, Bert M Drenth,
Anton W Ambergen, Jan-Willem Arends and Frederik BJM Thunnissen*

Abstract

The prognostic value of point mutations in codon 12 of the Kirsten ras (K-ras) gene in survival/clinical behavior of non-small cell lung cancer (NSCLC) is debated. The aim of this study was to examine the prognostic value of specific K-ras genotypes in patients with NSCLC using a point mutation detection method with a high sample sensitivity.

Patients with NSCLC who underwent curative surgery were selected. The histologic classification of 135 patients with NSCLC revealed 81 squamous cell carcinomas, 39 adenocarcinomas, 1 adenosquamous carcinoma and 14 large cell carcinomas. Associations of K-ras point mutations with clinical characteristics and survival were studied.

K-ras point mutations were detected in 33 of the 135 cases (24%). This percentage is explained by the 60% frequency of squamous cell carcinomas. Survival analysis showed prognostic significance of K-ras alterations on disease free survival ($P=0.04$) and an adverse effect of K-ras mutation on overall survival of patients with pathological stage II and IIIA tumors ($P=0.02$). The substitution of the wild type GGT (glycine) to GAT (aspartic acid) and ATT (isoleucine) showed a significantly worse prognosis compared with the wild type or other K-ras amino acid substitutions ($P=0.002$). Pathological stage and K-ras genotypes were independent unfavorable prognostic markers in NSCLC.

Our results suggest that specific K-ras codon 12 alterations may identify patients with a worse prognosis. Based on these data and a review of the literature we advocate a prospective study for adjuvant therapy in K-ras positive NSCLC.

1. Introduction

Lung cancer is the leading cause of cancer deaths in Europe and the United States^{5,256}, with the majority of patients dying from the disease within 2 years of diagnosis²¹⁸. For 1998, the trend will be that it stays the leading site of all cancer deaths in both men and women⁵. In addition, while 5-year survival rates continue to improve for most cancers, those for lung carcinomas stay poor⁴.

Lung cancer is a heterogeneous disease, being classified into two major groups: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC comprises about 75-80% of all lung cancer cases and consists of adenocarcinomas, squamous cell carcinomas and large cell carcinomas. For staging of SCLC the division in limited and extensive disease is used, while for

NSCLC the tumor-node-metastasis system is the basis for treatment and prognosis.

Outweighing all other prognostic factors, the existence of resectable early-stage disease is the most powerful predictor of outcome among patients with NSCLC¹²⁵. However, early recurrence of stage I patients is frequently observed and only one third of those patients are alive after 5 years²⁴. Thus, additional prognostic factors of long term biological behavior are necessary and could improve patient selection for additional therapeutic approaches.

With molecular diagnostic techniques several genetic alterations have been demonstrated in oncogenes and tumor suppressor genes. One frequently reported genetic alteration in NSCLC is the presence of point mutations in the Kirsten ras (K-ras) gene^{91,257}. Furthermore, studies have attempted to correlate K-ras alterations in lung cancer with clinical and prognostic features^{78,84,97,112,116,118,201,258}, sometimes with contradictory outcomes. Most of these studies were concentrated on only one subgroup of NSCLC, namely adenocarcinomas^{84,97,112,116,124} and used detection methods with relatively low sensitivity on sample level. In a previous study, we have demonstrated identification of K-ras alterations in squamous cell carcinomas, but to a lower extent than in adenocarcinomas²¹⁷. This finding was explained by the use of a highly sensitive method of point mutation detection, and is also supported by others^{104,199}. While K-ras gene alterations have been reported to predict survival in all stages of resectable NSCLC^{78,112,113,123}, other groups have pointed out that K-ras point mutations have prognostic value only in stage I tumors^{97,124}. Recent reports on K-ras genotypes and prognosis in NSCLC have further demonstrated a relationship between specific K-ras genotypes and prognosis^{122,123,201}.

The aim of this study was to examine whether specific point mutations in codon 12 of the K-ras gene are prognostically relevant in NSCLC. To this end, we used a rapid, reliable, highly sensitive method for detection of K-ras codon 12 point mutations in NSCLC, Point-EXACCT (point mutation detection using exonuclease amplification coupled capture technique)^{187,197,217}. The mutational data are discussed in relation to clinical parameters, possible lung pathogenesis, and the results reported by other investigators.

2. Materials and Methods

2.1 Patients and tumor specimens

The study population consisted of 135 patients with NSCLC who underwent curative surgery for NSCLC at the Maastricht University Hospital, Maastricht and De Wever Hospital, Heerlen between 1993 and 1997. The study also included 89 patients described previously whose tumors were

evaluated with different techniques for point mutations in codon 12 of the K-ras gene²¹⁷. All patient characteristics were extracted from the clinical files.

The tumor specimens were histologic classified according to the World Health Organization criteria²⁰⁰, which resulted in 81 squamous cell carcinomas, 39 adenocarcinomas including one bronchoalveolar carcinoma, 1 adenosquamous carcinoma, and 14 large cell carcinomas. The postsurgical pathological stage (pStage) of each tumor was classified according to the tumor-node-metastasis classification system²¹⁹.

For survival analysis, only those patients were selected from whom tumor samples from surgery specimens and follow-up data were available, resulting in a group of 110 patients. The study protocol was approved by the local ethical committee.

2.2 DNA isolation, K-ras gene exon 1 amplification and K-ras point mutation detection

Fresh tissue blocks were stored at -70°C after surgical resection. Histopathologic classification was performed on formalin-fixed, paraffin embedded and subsequently hematoxylin-eosin stained slides. DNA was isolated from 25 micrometer sections of tissue blocks using a rapid lysis procedure¹⁹⁷. DNA integrity and concentration was checked with agarose gel electrophoresis and ethidium bromide staining. K-ras coding sequences including codon 12 of the K-ras gene were amplified with primers of the first exon as described previously¹⁹⁷. The experimental details of the Point-EXACCT method for K-ras point mutation analysis were performed as described elsewhere¹⁹⁷.

Although several studies reported frequencies of K-ras alterations at other codons than codon 12, the majority of those alterations were found in codon 12^{97, 118, 124}. Therefore, we restricted our point mutation analysis to K-ras codon 12.

2.3 Statistical Analysis

Unifactorial analysis of various clinical and pathological factors was performed with chi-square analysis (χ^2 test). The Kaplan-Meier method was used to estimate the probability of survival as a function of time, and differences among the curves were computed using the log rank test. Mean and 75% survival times were calculated as indicators for prognosis since no value for median survival time was obtained for some of the subgroups during the observation period. Cox's regression analysis was performed to select independent factors affecting overall survival. Differences were considered to be significant when two-sided P-values were less than 0.05. Analysis of data was performed with the SPSS program package (SPSS Inc., The Netherlands).

The one case with adenosquamous carcinoma was for K-ras and survival analysis combined with the squamous cell carcinomas. The analyses with and without this case were similar.

3. Results

3.1 Detection of K-ras codon 12 point mutations

The spectrum of K-ras codon 12 mutations identified in the different histologic subtypes is shown in Table 1. Predominant point mutations were G-T transversions (21/33), of which wild type glycine was mostly converted to valine (15/21). Furthermore, two double mutations were identified, in which the wild type sequence for K-ras codon 12 GGT (glycine) was converted to ATT (isoleucine).

Table 1. Spectrum of K-ras codon 12 mutations found in NSCLC^a

Histology	AGT ser ^b	TGT cys	CGT arg	GAT asp	GTT val	GCT ala	ATT ile	No. of Mutations	No. of Patients
SqCC	3	0	0	0	7	0	0	10	82
Adeno	1	4	1	4	8	0	1	19	39
LCC	1	2	0	0	0	0	1	4	14
Total	5	6	1	4	15	0	2	33	135

^a the normal sequence for K-ras codon 12 is GGT, coding for glycine

^b Abbreviations: ser, serine; cys, cysteine; arg, arginine; asp, aspartic acid; val, valine; ala, alanine; ile, isoleucine

3.2 Clinicopathologic characteristics

Of the 135 patients analyzed in this study, the majority of patients (83%) were male. The mean age and standard deviation for men was 65 ± 9 years (range 41 to 81) and for women 60 ± 11 (range 36 to 80) years. All patients, except two were smokers or former smokers.

Clinicopathologic items with K-ras status of 135 patients and survival data are presented in Table 2. Mean survival time was 1355 ± 96 (range 0 to 2280) days. Five patients died within the first month after resection of the tumor. From the remaining patients the mean survival time was 1418 ± 96 (range 33 to 2280) days. Survival analysis was performed for all patients. Gender did not seem to affect the incidence of K-ras alterations. Mean age of

the patients with K-ras positive tumors (65 ± 7 years) did not differ ($P=0.39$) from mean age of patients with K-ras negative tumors (64 ± 10 years). Among different histologic subtypes difference in incidence of K-ras alterations in adenocarcinoma subtypes was highly significant compared with other histologic subtypes ($P<0.0001$).

3.3 Survival analysis

The population for survival analysis included 88 men and 22 women. Median age of the population was 66 years. When the patient group was divided in age 65 and >65 , a significant difference in overall survival was observed ($P=0.04$).

Stage of the tumor and nodal status were the only clinicopathologic variables highly significant for prognosis (figure 1). In addition, patients without node metastases fared well, with a mean survival of 1559 days, while patients with either hilar or mediastinal lymph node involvement showed remarkably shorter survival (table 2). Among different histologic subtypes, patients with an adenocarcinoma tend to have a slightly better prognosis than patients with the other subtypes, but this difference did not reach significance ($P=0.06$).

The overall survival time of all patients with and without K-ras point mutations stratified by pStage of the tumor and lymph-node-status, and disease free survival time were compared using Kaplan-Meier survival curves. Disease free survival time showed a significant difference ($P=0.04$) between K-ras positive and negative tumors (figure 2), while no difference existed between the two groups in overall survival time ($P=0.18$). Mean disease free survival was 1009 days for patients with K-ras positive tumors and 1787 days for K-ras negative tumors (figure 2). For patients with squamous cell carcinomas there was a trend toward shorter mean disease free survival time for K-ras positive tumors as opposed to K-ras negative tumors ($P=0.07$).

Figure 1. The Kaplan-Meier survival curve with respect to pStage. The survival curves are drawn for (a) pStage I, (b) pStage II, (c) pStage IIIA and (d) pStage IIIB-IV.

Table 2. Clinical information and K-ras status is shown in 135 NSCLC patients with univariate analysis of survival on the clinical items

Clinicopathologic characteristics	No. of Patients	K-ras positive		Mean survival (days)	75% survival (days)	P
		No.	(%)			
Age (years)						
≤65	60	14	(23%)	1458	490	0.04
>65	75	19	(25%)	1182	270	
Sex						
Male	112	27	(24%)	1289	282	0.24
Female	23	6	(26%)	1383	1215	
pStage						
I	77	20	(26%)	1639	746	<0.0001
II	39	7	(18%)	1097	332	
IIIA	10	3	(30%)	279	52	
IIIB-IV	9	3	(33%)	268	150	
Nodal status						
N0	94	23	(25%)	1559	360	<0.0001
N1	28	6	(21%)	885	330	
N2	13	4	(31%)	294	66	
Histologic type						
SqCC	82	10	(12%)	1300	304	0.06
Adeno	39	19	(49%)	1537	1215	
LCC	14	4	(29%)	678	52	
Smoking history ^a						
yes	133	33	(25%)	—	—	—
no	2	1	(50%)	—	—	

^a : not determined

Figure 2. The Kaplan-Meier survival curve of disease free survival time of all patients according to K-ras status. Patients with K-ras negative tumors (-) were compared with K-ras positive tumors (+).

As shown in table 3, overall survival time was significantly shorter for pStage II and IIIA patients who had K-ras positive tumors compared with the K-ras negative tumors ($P=0.02$). This pattern was also observed when only patients with squamous cell carcinoma subtypes were selected for survival analysis. For large cell carcinomas, patient groups were too small for further stratification by pStage. The analysis was also performed with stratification by nodal status which showed for patients with hilar lymph node metastases that mean survival for patients with K-ras positive tumors was significantly shorter than patients with the same nodal status without K-ras alterations.

Table 3. Prognostic value of K-ras codon 12 mutations in NSCLC patients with Point-EXACCT

Factor	K-ras Status	No. of Patients	Mean survival (days)	75% survival (days)	P
Histology: all					
pStage					
All	-	81	1430	312	0.18
	+	29	870	270	
I	-	48	1662	1260	0.75
	+	19	1187	510	
II	-	24	1222	450	0.01
	+	5	285	270	
IIIA	-	4	397	66	0.02
	+	2	44	35	
IIIB-IV	-	5	231	189	0.50
	+	3	328	150	
Node-status					
N0	-	60	1580	360	0.74
	+	20	1129	286	
N1	-	16	1042	330	0.03
	+	6	342	270	
N2	-	5	373	280	0.40
	+	3	163	35	
Histology: adenocarcinoma					
pStage					
All	-	15	1752	1260	0.24
	+	16	1020	270	
I	-	10	1801	-	0.27
	+	14	1047	286	
II	-	5	1385	1215	0.11
	+	2	315	270	
Histology: squamous cell carcinoma					
pStage					
All	-	58	1365	312	0.16
	+	9	712	304	
I	-	33	1628	746	0.75
	+	4	1468	1125	
II	-	17	1168	450	0.01
	+	3	252	24	
IIIA	-	4	397	66	0.05
	+	1	35	35	
IIIB-IV	-	4	219	16	0.71
	+	1	304	304	
Histology: large cell carcinoma					
pStage					
All	-	8	812	690	0.61
	+	4	990	990	

To assess whether the presence of a specific type of K-ras point mutation was associated with a worse prognosis, we compared overall survival for each type of K-ras alteration with the other mutations and with the overall survival of NSCLC patients without K-ras mutations (table 4). Patients with a positive K-ras status showed shorter overall survival time than K-ras negative tumors, but no significant difference was observed between both groups. When we analyzed overall survival by stratifying for different K-ras genotypes, two specific mutations showed a highly significant worse survival compared with the other mutations and with K-ras negative tumors: patients with a positive tumor for aspartic acid substitution or isoleucine showed the worst prognosis (figure 3A). When these two alterations were tested separately compared with other mutations, differences in survival were still significant. Above all, this difference remained significant when stratified by pStage of the tumor.

With regard to disease free survival time of different K-ras genotypes, highly significant differences were observed between all alterations identified ($P = < 0.0001$). When we divided different K-ras genotypes into three different groups including wild type, aspartic acid and isoleucine substitutions and the remaining mutations, mean disease free survival time for patients with the wild type K-ras was 1787 days, for aspartic acid and isoleucine mutation 310 days and for the remaining K-ras alterations 1072 days ($P = 0.02$), (figure 3B). Further separation between the two different mutation groups did not reveal a significant difference in disease free survival time ($P = 0.19$).

Table 4. Univariate analysis of survival in different K-ras genotypes

Factor	No. of Patients	Mean survival (days)	75% survival (days)	P
K-ras mutation				
-	81	1430	312	0.18
+	29	870	270	
-	81	1430	312	0.002
GAT+ATT	6	237	51	
other	23	1013	304	
GAT+ATT	6	237	51	0.01
other	23	1013	304	
GAT	4	271	14	0.08
other	25	942	286	
other (- ATT)	23	1013	304	0.048
ATT	2	169	52	0.047
other	27	925	270	
other (- GAT)	23	1013	304	0.02

Figure 3. The Kaplan-Meier survival curves of different K-ras genotypes. (A) Overall survival time of different K-ras genotypes. (B) Disease free survival time of different K-ras genotypes. Patients with (a) wild type for K-ras were compared with (b) other K-ras mutations than (c) ATT (isoleucine) and GAT (aspartic acid) mutation.

Cox's regression analysis was performed to identify the most powerful prognostic factors. The hazard ratios were calculated with two models using clinicopathologic variables interrelated with K-ras mutations. The following factors were entered into the first model: age, gender, pStage, K-ras mutation and histologic type. Therefore, a second model was used which combined pStage and K-ras mutation. Using only pStage and K-ras mutation in the second model, the hazard ratio and 95% confidence interval for the K-ras GAT and ATT mutations versus other mutations was 10.11 (2.63-38.81) and for pStages II-IV versus pStage I was 10.61 (2.96-38.02), (table 5). With regard to disease free survival, pStage and K-ras mutation were still significant prognostic variables according to Cox's proportional hazard model (data not shown).

Table 5. Multivariate analysis of prognostic factors in NSCLC

Variable	Categories compared ^a	Hazard Ratio ^b	95% CI	P
pStage	II-IV vs. I	13.83	3.47-55.14	<0.0001
K-ras	GAT and ATT vs. other	9.69	2.22-42.34	0.005

^a For each variable, the prognostic significance of the first category listed was assessed by comparing the category with the reference category (second category listed)

^b The regression coefficients (rc) were converted to hazard ratios by computing $\exp(rc)$. The 95% confidence interval (CI) for the hazard ratio was computed as $[\exp(rc - 1.96s.e.), \exp(rc + 1.96s.e.)]$, where s.e. was an estimated standard error of rc

4. Discussion

In this study the prognostic value of specific K-ras point mutations in relation to clinicopathologic variables was examined in patients with NSCLC. As expected, pStage is the most important prognostic factor. Apart from pStage, the presence of K-ras point mutations was associated with a significantly shorter disease free survival time. In patients with locoregional lymph node metastases K-ras alterations presented with an overall worse outcome. Importantly, the presence of specific K-ras mutations showed significant difference in overall and disease free survival time. Also in the multivariate analyses the presence of specific K-ras mutations was beside pStage the only significant prognostic factor.

The results of the 135 NSCLC patients analyzed for point mutations in codon 12 of the K-ras gene with the highly sensitive technique Point-EXACCT

revealed a high frequency (49%) of K-ras alterations in adenocarcinomas and a particularly high mutation frequency (12%) in squamous cell carcinomas. This finding corresponds to the results of several previous reports, which demonstrated the identification of K-ras alterations more frequently in adenocarcinoma than in squamous cell carcinoma subtypes^{81,93,95}. However, the frequencies are substantially higher than previous estimations, which have been based on the technical analysis with a relatively low sample sensitivity. The variations in frequencies of K-ras alterations in several reports may at least in part be explained by differences in sensitivity of point mutation detection methods. The higher prevalence of K-ras alterations in squamous cell carcinomas in our study is due to the high sensitivity of our method, which has a detection limit far beyond conventional techniques such as direct sequencing of amplification products²¹⁷. In addition, the majority of K-ras alterations identified in squamous cell carcinoma subtypes represent low fraction mutations²¹⁷, an observation made by other groups as well¹⁹⁹. Also, the high sensitivity of our method was successfully proven for identification of K-ras alterations in sputum of patients with adenocarcinoma of the lung²⁴¹. Although a highly sensitive method was used for K-ras codon 12 analysis, the overall mutation frequency of 24% in this study on NSCLC was not higher than frequencies in other studies^{78,118}, and is explained by the presence of 60% squamous cell carcinomas in this study.

Our results also extend previous observations relative to different spectrum of K-ras alterations among histologic subtypes. Predominant K-ras codon 12 alterations were G-T transversions, as found in other reports^{84,97,113,124}. However, other studies also demonstrated majority of mutations as G-A transitions, sometimes accompanied by occurrence of these mutations in squamous cell carcinomas^{118,123}.

Among the 135 tumors analyzed, we found one patient with an adenocarcinoma and another patient with a large cell carcinoma with a double mutation, GGT to ATT in K-ras codon 12, which transforms the wild type glycine to isoleucine. The presence of double mutations in K-ras codon has also been found by others, identifying the substitution from glycine by phenylalanine^{95,104}.

A literature survey on studies of K-ras and prognosis in NSCLC is summarized in table 6. The finding of pStage as the most important prognostic factor in our population of NSCLC patients has been reported in many other studies^{84,116,259}. Apart from this result, previous studies on NSCLC cases have also demonstrated a significant correlation between K-ras alterations and unfavorable prognosis (table 6). While first reports on K-ras point mutation detection and prognosis were mainly focussed on the occurrence of K-ras alterations in adenocarcinomas^{84,97,112,116,124}, other studies used all histologic subtypes^{78,104,113,118,120,123,201}. In most studies, survival probabilities were stratified according to different stages of disease. Patients with K-ras positive tumors had a significantly shorter disease free survival time, while no significant

difference in overall survival time was found between K-ras positive and negative cases. Interestingly, the presence of K-ras point mutations associated with shorter disease free survival time in patients with squamous cell carcinoma denotes a possible role of K-ras alterations in this histologic subtype as well.

The observation that K-ras shows prognostic significance in patients with lymph node metastases denotes a possible association with metastatic properties of the tumor and the presence of K-ras alteration as prognostic marker (table 3): i.e. when other genomic perturbations occur, the metastatic potential of the tumor could be enhanced by the presence of K-ras mutations. This finding is supported at least in part by the results of Cho and coworkers, who demonstrated an association between the presence of K-ras and poor prognosis in patients with stage III disease, but not in a subset of patients with stage I-II disease¹¹⁸. However, other groups have shown contradictory results, demonstrating an adverse effect of K-ras mutation on survival restricted to patients with node-negative status or stage I disease^{97,124}. This difference may be explained by the fact that Cho and coworkers and our study used all histologic subtypes of NSCLC for analysis, while the other studies only investigated adenocarcinomas.

The relationship of K-ras and prognosis in NSCLC has been subject of many published reports. In this study, we present an update of the manuscripts reported on this issue (table 6). In the majority of these reports, an adverse effect of K-ras alterations on the clinical course was demonstrated. If a sensitive technique is used, then the prognostic finding can be extended from adenocarcinoma to all NSCLC. Altogether, these findings denote a prognostic relevant role of K-ras in NSCLC and it is to our opinion worthwhile to examine the presence of K-ras alterations as a marker for adjuvant therapy in a prospective study.

The occurrence of both aspartic acid and isoleucine amino acid changes was also significantly related to diminished survival. This is in agreement with the findings of others, in which individuals with a G-A transition in position 2 of K-ras codon 12 carried a worse prognosis than the other mutations^{84,122,123,201}. Kern and coworkers demonstrated G-A transitions in base 2 of K-ras codon 12 as predominant alteration in adenocarcinoma of the lung and found a slightly higher hazard ratio, but did not achieve statistical significance⁸⁴.

Regarding a possible genotypic classification of lung carcinomas so far no clear distinctions have been observed between different studies. While several reports have described aspartic acid substitutions with worse outcome^{84,122,123,201}, Keohavong and coworkers showed a worse prognosis of the valine and arginine mutations compared to wild type or other amino acid changes, but statistical significance was not reached⁹⁵.

So far, no consistent explanation has been put forward for the biological properties of different K-ras codon 12 genotypes. Based on the results of transfection assays, Seeburg and coworkers demonstrated differences in

transformation properties of several genotypes of the Harvey ras (H-ras) gene, another member of the ras family²⁶⁰. Mutations which give rise to gene products with the most potent transforming ability (measured by colony morphology) were those encoding valine, leucine, isoleucine, arginine and threonine at codon 12 of the H-ras gene. The intermediate class of mutants was represented by serine, methionine, cysteine, tyrosine, phenylalanine, tryptophan, histidine, aspartate, glutamate, alanine and asparagine at H-ras position 12. Mutants with the most subtle transforming activity were lysine and glutamine at position 12²⁶⁰.

Another possibility underlying the negative relationship between aspartic acid and isoleucine substitutions of K-ras codon and prognosis may be that aspartic acid and isoleucine substitutions show different GTPase activity relative to the other K-ras mutations. Mutations of K-ras codon 12 result in a defect in their intrinsic GTP hydrolysis as well as GTP hydrolysis accelerated by GTPase activating proteins. A previous study on the neuroblastoma ras p21 protein demonstrated a reduction in the GTPase activity of the aspartic acid substitution for codon 12, while another mutant such as valine showed decrease in the GTPase activity, despite equal transforming potency²⁶¹.

An interesting finding was that the two K-ras alterations leading to aspartic acid and isoleucine mutation were identified in patients with adenocarcinoma and large cell carcinoma, but not in squamous cell carcinoma, as found by other groups^{80,81,84,95,262}. However, other studies demonstrated aspartic acid changes in the latter subtype^{118,123}. So geographical differences may exist in frequency, type and prognostic significance of K-ras mutations. As mentioned earlier, differences in frequencies of mutations between different histologic subtypes and corresponding prognostic significance could rely on the sensitivity of the methods used for detection of point mutations.

In conclusion, our results suggest that specific K-ras codon 12 alterations may identify patients with a worse prognosis. Based on these data and a review of the literature we advocate a prospective study for adjuvant therapy in K-ras positive NSCLC.

Table 6. Prognostic value of K-ras gene mutations in NSCLC patients

Histology ^a	pStage	No. of patients	Mutations No. (%)	P ^b	Sens. ^c	Study
1. adeno	I-III A	69	19 ^d (28)	11	1/10	Slebos ¹¹²
2. adeno	all	115	18 (16)	-	1/10	Sugio ⁹⁷
	I	52	8 (15)	11		
3. adeno	I-III A	44	16 ^e (36)	-	1/10	Kern ⁸⁴
		44	6 ^f (14)	11		
4. adeno	I	30	7 (23)	-	1/10	Isobe ¹¹⁶
5. adeno	all	109	33 ^e (30)	-	1/25	Silini ¹²⁴
	I	81	23 ^e (28)	1		
6. adeno	III-IV	62	16 (26)	-	>1/100	Rodenhuis ²⁶⁵
7. adeno/l.c.	all	103	33 (32)	1	1/10	Nemuncitis ²⁶⁶
	all	103	14 ^f (14)	1		
	all	103	8 ^g (8)	1		
8. all	I-IV	66	19 (29)	-	1/10	Mitsudomi ⁷⁹
	I-III A	21	8 (38)	1		
	IIIB-IV	45	11 (24)	-		
		45	6 ^e (13)	1		
9. all	I-III A	66	13 (20)	1	1/20	Rosell ¹¹³
10. all	all	192	51 (27)	-	1/10	Rosell ¹²³
	IIIA	72	5 ^h (7)	1		
11. all	all	275	57 ⁱ (21)	1	1/10	Rosell ²⁰¹
	IIIA	102	8 ^h (8)	1		
12. all	I-III B	58	14 (24)	1	1/10	Cho ¹¹⁸
	IIIA	24	18 ^e (75)	1		
	I-II	31	26 ^e (84)	-		
13. all	all	159	11 ^d (7)	1	1/10	Fukuyama ¹
	I-II	107	8 ^d (7.5)	1		
14. all	all	126	39 ^j (31)	-	1/20	Keohavong ¹
	all	173	14 ^k (8)	-		
15. all	all	144	12 (8)	1	1/10	Huang ²⁵⁷
16. all	all	101	19 (19)	-	1/10	Greatens ²⁵⁸
17. all	all	110	29 ^d (26)	1	1/15,000	this stud
		110	6 ^l (5.5)	1		
	II-III A	35	7 ^d (20)	1		

^a adeno, adenocarcinoma; l.c., large cell carcinoma^b -, p < 0.05: worse prognosis K-ras; p < 0.005: worse prognosis K-ras; —, no significant association with survival^c estimation of sample sensitivity (Sens.) of the method used^d point mutation analysis on codon 12 only

- ^e point mutations detected in codon 12 only
- ^f c-erbB2 expression plus K-ras mutation
- ^g strong p21 expression plus K-ras mutation
- ^h aspartic/serine mutation
- ⁱ study also included 66 patients described in No. 9
- ^j survival for adenocarcinoma plus adenosquamous carcinoma
- ^k valine/arginine mutation
- ^l aspartic/isoleucine mutation

Nine

Epilogue

1. Introduction

In the present chapter firstly, relevant technical aspects regarding point mutation detection are mentioned with future options. Secondly, the role of K-ras point mutation detection in NSCLC with Point-EXACCT is discussed. Finally, suggestions for future research towards early detection are presented in a broader context.

2. Technical aspects

2.1 Development of Point-EXACCT

Using the K-ras gene as a model system, the development of the Point-EXACCT technique was described in this thesis. The Point-EXACCT technique was designed for analysis of single base substitutions, of which the exact place of the nucleotide sequence in the gene for analysis is known. K-ras point mutations have been described to occur in base 1 and 2 of K-ras codon 12, of which the normal sequence corresponds to GGT. The optimization of different critical steps in the Point-EXACCT method was based on the analysis of 7 different cell lines with a known sequence for the K-ras codon 12 region: HL60, H716, A549, Calu-1, CFPAC-1, SW480, and SW1116. In figure 1, the relative optical density values of 6 different cell lines HL60, A549, Calu-1, CFPAC-1, SW480, and SW1116 obtained by Point-EXACCT are presented. Cell line H716 was also used as a control for the wild type sequence GGT for K-ras codon 12, of which the relative optical density values corresponded to those of cell line HL60 (data not shown).

Figure 1. The relative optical density values obtained by Point-EXACCT are shown for 3 cell lines tested for base 1 of K-ras codon 12, and for 3 cell lines with a K-ras mutation for base 2 of codon 12. Cell line HL60 only showed a signal after incubation with the wild type probe G. The GGT to AGT mutation was detected in cell line A549. Calu-1 showed both wild type and mutated T sequence for base 1 of K-ras codon 12, referring to a heterozygous mutation. For base 2 of K-ras codon 12, cell line SW480 was positive for the mutated T sequence and CFPAC-1 showed a positive signal after incubation with probe A, whereas cell line SW1116 demonstrated both wild type and mutated C sequence for base 2 of K-ras codon 12. These cell lines were used as standard controls during all Point-EXACCT experiments.

The Point-EXACCT method has been validated 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. The results show that exactly the same alterations identified with sequencing were also detected with Point-EXACCT. However, with Point-EXACCT a higher number of K-ras alterations were identified, emphasizing the high sensitivity.

Beside point mutations of K-ras codon 12, several other genetic disorders caused by known point mutations can be diagnosed by Point-EXACCT. Currently, this method is being tested for detection of point mutations of the p53 gene in patients with head-neck tumors (V. van Houten, personal communication).

2.2 Sensitivity of Point-EXACCT

In order to test the sensitivity of the Point-EXACCT method, reconstruction experiments were performed for cell lines with a mutation for both base 1 and 2 of K-ras codon 12. In addition, the converse experiment, in which cells with the wild type sequence for K-ras codon 12 were diluted in cells with a mutation was also performed. DNA extracted from cell mixtures containing a known ratio of cells with a K-ras alteration in cells with the wild type sequence have been tested many times starting with a new amplification and point mutation detection reaction. Using 1 μg DNA in 100 μl for the amplification reaction, 1 cell containing a K-ras mutation in an excess of 15,000 wild type cells could be detected. Since Point-EXACCT uses 8.5 μl amplification product after a PCR reaction containing 1 μg DNA in 100 μl amplification reaction mix, and assuming a DNA content of 5-7 pg per cell, amplified DNA of approximately 12,000-17,000 cells per well is used at the start of the Point-EXACCT hybridization reaction. In an attempt to investigate the maximum sensitivity, up to 4 μg DNA was used in the amplification reaction. Using this high concentration of DNA a signal could be obtained for the specific mutation at the 1:78,000 dilution, demonstrating the detection of 1 cell containing a mutation amidst more than 75,000 wild type cells. Theoretically in this setting DNA of about 50,000 cells is present in each well. Thus, in practice, the presence of DNA from a single cell with a mutation in the reaction tube will be detected with Point-EXACCT.

2.3 Threshold values

Based on the data obtained from several different reconstruction experiments, threshold values for the Point-EXACCT assay have been determined. The threshold line of the background values was set at the mean plus three times the standard deviation. For these optical density values the one sided maximal risk of false positivity is 0.5% at threshold level. For higher relative optical density values this risk of false positivity is dramatically reduced.

A clinical sample was considered positive when the relative optical density signal exceeded twice the following values: 1) the relative optical density signal of the control (a mixture of one cell with a mutation between 15,000 genetically normal cells) as measured that day, and 2) the mean relative optical density signal of all positive control specimens.

Because no cell line was available which contained the GGT to CGT mutation, positivity levels were based on the mean plus three times the standard deviation of the background products.

In general, the Point-EXACCT method was performed twice for all specimens analyzed for the presence of alterations in base 1 and 2 of K-ras

codon 12. Discordant specimens were analyzed three times. As a result, a specimen was only considered positive when a positive signal was obtained in more than half of the experiments. A false-positive result can be obtained by DNA-polymerase induced errors during the amplification reaction or by inaccurate performance of the Point-EXACCT procedure by the laboratory personnel. The replication fidelity of Taq DNA polymerase, the enzyme used for the amplification reaction has been determined using a PCR-based forward mutation assay, with an average error rate (mutation frequency/bp/duplication) of 8.0×10^{-6} .²⁰⁷ From an estimated number of more than 2000 Point-EXACCT measurements of 404 clinical specimens used in this study, 21 (1%) measurements were once positive for a specific mutation, whereas in the following 2 measurements a negative signal for the specific mutation was obtained. Ultimately, these specimens were classified as negative for a specific mutation.

Point-EXACCT is a technique which requires accuracy, and the ligation step is crucial. With each probe combination, the ligation step necessitates optimization. Once the conditions are set, Point-EXACCT can be performed many times.

2.4 Technical improvements of Point-EXACCT

In a recent editorial it has been mentioned that the Point-EXACCT method may be further improved by the use of small peptide nucleic acid probes (PNAs) during amplification. Such probes suppress amplification of wild type alleles and consequently increase the relative ratio of mutant to wild type sequences²⁶⁴. However, this remains to be proven. Another adaptation of Point-EXACCT is the use of an enriched amplification reaction, in which a thermostable restriction endonuclease BstN1 is incorporated as part of the amplification reaction. The concurrent activity of the restriction endonuclease BstN1 and Taq DNA polymerase further allow simultaneous amplification of the mutant sequences and inhibition of amplification of wild type K-ras³⁰¹. In addition, the use of a chemiluminescent assay or fluorescent dyes attached to the hybridization probes may give stronger signals. Presently, the detection is in the picomolar range. The entire process, including the suggested improvements, may be easily automated and adapted for use in a routine clinical laboratory. The possible inaccuracies in performance are likely to be overcome.

In future, the Point-EXACCT method could be developed as a chip-based assay. This development includes a merge of microjet technology with other biotechnology approaches. The level of detection is then likely to be in the attomolar range, requiring less amplification of target DNA. The time of analysis can be reduced to a few hours, allowing high throughput. In addition, other genetic alterations, such as p53 could be examined simultaneously by

using different detection probes. The assay can then be used for evaluation of multiple markers at a time.

3. The role of K-ras point mutation detection in NSCLC with Point-EXACCT

In this thesis, the role of K-ras point mutation detection in NSCLC was investigated. Point mutations occurring in the K-ras gene were selected, because they represent the most frequently occurring individual point mutations in lung carcinomas. Cells with a point mutation have the advantage that they can be detected against the background of a large population of normal cells in contrast to e.g. amplifications and deletions.

Compared with literature data obtained from conventional methods, which only enable detection of high fraction mutations, a higher frequency of K-ras alterations among different histologic subtypes was obtained with Point-EXACCT. This is explained by the presence of low fraction mutations, mainly in patients with squamous cell carcinoma. Among different histologic subtypes examined with Point-EXACCT, K-ras alterations were identified in 11-15% of squamous cell carcinomas, 35-50% of adenocarcinomas and 29-38% of large cell carcinomas.

Using the Point-EXACCT assay for identification of K-ras alterations in archival sputum specimens derived from patients who later went on to develop lung cancer, the role of the K-ras gene as an early marker in lung cancer was demonstrated. If, in future, molecular techniques will be used to prospectively screen sputum samples in people at risk for lung cancer, K-ras mutations may be detected, while on X-ray and CT-scan no tumor is visible. Because (1) biologically, K-ras is found just before the visible occurrence of lung cancer in animal studies, and (2) in humans is a relatively late event in lung carcinogenesis, we feel that, when techniques with a high specificity are used, these people should be treated according to the guidelines for occult lung cancer of the American Thoracic Society and European Respiratory Society²²⁵. This statement is further strengthened by our results, which indicate that the presence of K-ras alterations is specifically related to tumor formation and further suggest that these changes do not occur on a widespread basis in the normal bronchial epithelium. We further point out that no K-ras alterations were detected in any bronchial wash specimens from patients without oncologic disease. Altogether, these data denote both a high specificity not only for the Point-EXACCT assay, but also for K-ras as a specific biomarker, which is a useful feature for early lung cancer detection.

Apart from a role of the K-ras gene in early diagnosis of malignancy, we have demonstrated the clinical use of the K-ras gene in diagnosis of peripheral lung tumors. On the basis of our findings, we suggest that patients, with a

suspect peripheral lung tumor on the X-ray of the thorax and the presence of a K-ras positive bronchial wash specimen, in the absence of a cytologic or histologic diagnosis, should nevertheless be considered for curative surgery. These data uncover a possible role of molecular analysis as an important adjunct to bronchoscopy in lung cancer diagnosis.

Another (unpublished) possibility of Point-EXACCT application in lung cancer is its use in pathology for differential diagnosis of patients with a second tumor, where the question is the presence of a lung metastasis of e.g. breast carcinoma, or a second primary lung cancer.

In this thesis, the importance of specific K-ras alterations as genetic marker of clinical outcome has been revealed. Although such specific mutations with a worse clinical outcome only involve a minority of NSCLC patients, this finding has been confirmed in other studies and therefore, may be a useful criterium for adjuvant therapy in this particular subset of patients.

4. Suggestions for further research towards early detection

In the past, the screening programs using roentgenography and sputum cytology have not been successful approaches to early lung cancer detection. Primary prevention is the most desirable solution for lung cancer, but despite the efforts of education and anti-smoking campaigns it is not likely that the exposure to tobacco smoke will entirely disappear during the next century. Therefore, secondary prevention is likely to still have a major place in the fight against lung cancer. In the present heading, several approaches for screening are discussed including 1) identification of high-risk groups, 2) the use of molecular diagnostic techniques to detect genetic alterations and 3) a new array of diagnostic imaging techniques. In addition, suggestions regarding the future use of Point-EXACCT in cancer research are presented.

4.1 Identification of high-risk groups

A priority for screening approaches is to ensure that the sensitivity of the process is high while the cost of screening is reasonable. Because the probability of development of lung cancer in the general population is typically less than 0.001%, screening of the whole population is not worthwhile. Therefore, it is useful to identify populations with an increased risk of development of lung cancer²⁶⁵. The predominant risk factor associated with lung cancer is smoking²⁶⁶. Additional environmental carcinogens that increase the risk for lung cancer and which may act synergistically with active smoking include passive smoke exposure²⁶⁷, residential radon^{268,272}, other pollutants in the urban air, known as polycyclic hydrocarbons and diesel engine

exhaust^{160,273,274}, and occupational exposure to carcinogens such as asbestos, chromate, nickel, beryllium, hydrocarbons, sulphuric and hydrochloric acid, nitrogen mustard gas, acetonytrile, chloromethyl ethers, vinyl chloride, arsenic, cadmium, uranium, and radon^{156-161,275-281}. Other risk factors for lung cancer are genetic predisposition²⁸²⁻²⁹⁰, chronic obstructive pulmonary diseases (COPD)²⁹¹⁻²⁹⁴, and familial lung cancer with specific germline mutations^{73,295,296}.

4.2 Molecular diagnostic techniques

The development of molecular diagnostic techniques to unravel genetic alterations during the preclinical phase of lung cancer may provide an improvement in the early detection of lung cancer. The analysis of K-ras point mutation detection alone for early lung cancer diagnosis in e.g. sputum specimens may theoretically be useful for 15% of all lung cancer patients, provided that tumor cells are present in the specimen. Therefore, investigating more potential biomarkers for lung cancer such as p53 is mandatory. P53 mutations occur in 50-70% of all lung carcinomas, of which 50% are point mutations^{116,297,298}. Using a combination of the K-ras and p53 genes, the sensitivity of point mutation detection in lung cancer diagnosis may be increased up to 40-45%, based on the principle of Point-EXACCT alone. This would enable detection of both NSCLC and SCLC patients, since K-ras alterations are predominantly found in NSCLC and p53 mutations occur in approximately 50% of SCLC. Clinical specimens of high-risk patients, e.g. COPD can be analyzed for the presence of these genetic alterations.

4.3 A new array of diagnostic imaging techniques

Nowadays, a new array of non-invasive diagnostic imaging techniques is available which can visualize tumors up to 0.3 to 0.5 cm. Helical or spiral computed tomography scan is useful for imaging lung cancer because it is done in one continuous motion rather than as radiographic slices. This means a complete image can generally be obtained in a single breath-hold¹²⁹⁹. About 30% of scans will find an indeterminate nodule, although its benign nature can often be determined by typical calcifications around the nodule, or by the presence of fat¹⁷¹. In a recent report, computed tomography was compared with conventional chest radiography more accurate in mass screening for lung cancer and led to early detection and accurate diagnosis of lung cancer³⁰⁰.

Positron Emission Tomography scanning, with its display of glucose uptake in tumors, is also a promising approach for evaluation of solitary nodules and lymph nodes, because tumor cells with their rapid turnover rate have higher glucose needs than normal cells.

4.4 Towards a strategy for early detection

The development of the Point-EXACCT method as a chip-based assay could enable evaluation of multiple genetic markers. Since p53 mutations are scattered among different exons, sufficient information can be obtained on the occurrence of these alterations during carcinogenesis of bronchial lining cells. Once the assay is optimized for evaluation of multiple genetic markers at a time, this method could be generally used for other malignancies as well, such as colorectal or pancreatic cancers. The sensitivity of the chip-based assay on two genes is relatively low (40-45%) for lung cancer, but the specificity is high. This method can be combined with other markers on sputum such as an antibody approach.

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Ten

Summary / Samenvatting

Summary

Lung cancer has a high incidence and mortality. Early detection programs with conventional methods such as X-ray and sputum cytology have failed to improve mortality. Lung carcinomas are now considered as a genetic disease. Many regions in the genome have been thought to contain candidate genes related to the development of lung cancer. The mutations in p53 and K-ras genes are the best characterized in lung cancer and are thought to occur late in the development of lung cancer. Therefore, new approaches that use genetic alterations such as K-ras as potential biomarkers may be beneficial for early detection of lung cancer.

Previously, several molecular methods have been utilized for the detection of K-ras point mutations in lung cancer. However, difficulties in critical analytic steps following DNA amplification have prevented the technology from being more widely applied in clinical laboratories and the biotechnology industry.

For early detection of K-ras mutations in a heterogeneous cell population such as sputum or bronchial wash specimens, where only a small fraction of tumor cells containing the mutation are present among much larger numbers of genetically normal cells, a highly sensitive technique is required, which should be very specific but not time-consuming, technically simple and safe.

Chapter One comprises a general introduction to the field of study. Highlighted are genetic alterations occurring in the multistep process of lung cancer development. It also presents a historical overview on the subject of lung cancer screening. The aims of the present study are further discussed.

Chapter Two describes the technological development of a new technique, Point- EXACCT, for detecting the presence of a specific nucleotide sequence within a double-stranded DNA in a sample comprising: a. digestion of the double-stranded DNA with an exonuclease which converts at least a portion of the double-stranded DNA to single-stranded DNA; b. hybridization of the single-stranded DNA with i) a first probe adapted with biotin which can be captured to a solid support (streptavidin), and ii) a second probe labeled with digoxigenin which can hybridize with the single-stranded DNA adjacent to the hybridized first probe; c. capturing the biotin on the first probe hybridized to the DNA to the streptavidin; d. ligation of the hybridized first and second probes in cases of perfect complementarity between probes and template DNA; e. denaturation of the ligated first and second probes from the hybridized single-stranded DNA; f. removal of uncaptured labeled digoxigenin probe; and g. detection the presence of captured detectable digoxigenin, which indicates the presence of the specific nucleotide sequence within the double-stranded DNA in the sample.

Solution hybridization is an essential step in sequencing and some point mutation detection methods. In practice this hybridization is hampered resulting in the need of additional purification of amplification products. In **Chapter Three**, the effect of pretreatment with exonuclease on different point mutation detection methods is analyzed. With the use of exonuclease, the detection of heterozygosity using fluorescent cycle sequencing is becoming more reliable. The high sensitivity of Point-EXACCT due to the use of exonuclease makes it a highly promising method for large-scale screening of (pre)malignant changes in patients with a high risk for cancer development.

In **Chapter Four**, the Point-EXACCT method is validated for detection of K-ras point mutations in a large series of NSCLC samples by comparing the results obtained with Point-EXACCT with two sequencing methods. 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. The finding of K-ras mutations in squamous cell carcinomas is explained by the high sensitivity of the Point-EXACCT method. Therefore, Point-EXACCT may be applicable for detection of those alterations occurring at a low frequency among an excess of cells with wild type DNA.

In **Chapter Five**, using the Point-EXACCT assay for identification of K-ras alterations in archival sputum specimens derived from patients who were diagnosed with lung cancer, the role of the K-ras gene as an early marker in lung cancer is investigated. DNA from paraffin-embedded adenocarcinoma and corresponding sputum samples of 22 patients was analyzed for mutations of the K-ras gene. A mutation in K-ras codon 12 was shown in more than half of the tumor samples, and in 45% of the patients with a K-ras mutation in the tumor, the same type of mutation was identified in at least one sputum sample. No K-ras alterations were found in sputum samples from patients with a K-ras negative tumor. Time between K-ras point mutation detection in sputum and clinical diagnosis of lung cancer varied from one month to almost 4 years. From this study we conclude that Point-EXACCT is suitable for detection of K-ras point mutations in sputum samples of patients with adenocarcinoma of the lung. This procedure may be an important adjunct to cytology in the early diagnosis of lung cancer since K-ras point mutations were found in sputum specimens that were diagnosed negative for malignancy by cytological analysis.

Chapter Six investigates whether K-ras point mutations also occur in nonmalignant bronchial epithelium. To this end, the bronchial epithelium of 75 patients who were treated for NSCLC with curative surgery was analyzed for the presence of K-ras point mutations. Analysis was performed on both tumor specimens and nonmalignant bronchial brushes obtained from an area distant from the tumor location. The finding of K-ras alterations in tumor specimens but not in normal bronchial epithelial cells collected at distant areas from the tumor supports the hypothesis that the occurrence of K-ras point mutations is

specifically related to tumor. These data may lead to a refinement of the concept of field cancerization and may suggest that a preferential susceptibility of certain cell types may exist for specific carcinogens.

In **Chapter Seven**, the additional diagnostic value of K-ras point mutations in bronchial wash specimens of patients under suspicion of lung cancer is studied. A consecutive series of bronchial wash specimens without a cytologic diagnosis of malignancy was investigated with Point-EXACCT in patients with abnormalities on X-ray and in a control group. Point mutation analysis of K-ras codon 12 with Point-EXACCT was performed in bronchial wash specimens and corresponding tumor tissues, if available. With Point-EXACCT K-ras alterations were identified in 21% bronchial wash specimens without a decisive diagnosis of malignancy. No K-ras codon 12 alterations were identified in bronchial wash specimens of patients without lung cancer. The presence of K-ras alterations in bronchial wash specimens is clinically useful in diagnosis of peripheral tumors and molecular analysis can serve as an important adjunct to bronchoscopy in lung cancer diagnosis.

In **Chapter Eight** the prognostic value of specific K-ras genotypes in patients with NSCLC is examined. Associations of K-ras point mutations with clinical characteristics and survival were studied. Survival analysis showed prognostic significance of K-ras alterations on disease free survival and an adverse effect of K-ras mutations on overall survival of patients with pathological stage II and IIIA tumors. The substitution of the wild type GGT (glycine) to GAT (aspartic acid) and ATT (isoleucine) appeared to be a mutation type showing significantly worse prognosis compared with the wild type or other K-ras point mutations. We suggest that specific K-ras codon 12 alterations may identify patients with a worse prognosis, who therefore must be candidates for adjuvant therapy. Based on these data and a review of the literature we advocate a prospective study for adjuvant therapy in K-ras positive NSCLC.

In **Chapter Nine** the technical aspects of Point-EXACCT with limitations and improvements have been discussed as well as the outlines for further a strategy towards early detection of lung cancer.

Samenvatting

Longkanker gaat gepaard met een hoge incidentie en mortaliteit. In het verleden verricht onderzoek naar vroegdetectie van longkanker met behulp van röntgenfoto's en sputumcytologie leidde niet tot vermindering van de mortaliteit. Tegenwoordig beschouwt men longkanker als een ziekte waarbij veranderingen in de nucleïnezuren optreden. Verschillende gebieden in het genoom bevatten kandidaatgenen die gerelateerd zijn aan het ontstaan van een longtumor. Het best gekarakteriseerd zijn mutaties in het p53 en het K-ras gen. Beiden ontstaan laat in het ontwikkelingsproces van longkanker. Nieuwe technieken voor de detectie van genetische veranderingen, zoals K-ras mutaties kunnen mogelijk een bijdrage leveren aan de vroegdetectie van longkanker.

Tot op heden zijn er reeds verschillende moleculaire methoden ontwikkeld voor de detectie van K-ras puntmutaties bij longcarcinomen. Deze methoden hebben een relatief geringe gevoeligheid en worden niet standaard toegepast in de kliniek en de biotechnologische industrie.

Vroegdetectie van K-ras mutaties in een heterogene celpopulatie zoals in sputum of bronchusspoelsels met slechts een kleine fractie tumorcellen tussen de meerderheid 'genetisch normale' cellen, eist een zeer gevoelige methode die zeer snel, specifiek, technisch eenvoudig en veilig is.

Hoofdstuk Een geeft een algemene inleiding over het onderzoek. Belangrijke aandachtspunten zijn de beschrijving van de verschillende genetische veranderingen die optreden tijdens het meerstapsproces van longkanker ontwikkeling. Een historisch overzicht over longkankerscreening wordt eveneens gepresenteerd. Verder worden de doelstellingen van de huidige studie aangegeven.

Hoofdstuk Twee behandelt de technologische ontwikkeling van een nieuwe methode, genaamd Point-EXACCT. Deze methode wordt gebruikt voor de detectie van een specifieke nucleotide variatie in een dubbelstrengs DNA sample door: a. het dubbelstrengs DNA te knippen met een exonuclease enzym dat een deel van het dubbelstrengs DNA omzet in enkelstrengs fragmenten; b. het enkelstrengs DNA te hybridiseren met i) een eerste probe die gelabeld is met biotine wat vervolgens aan een vaste matrix (streptavidine) gekoppeld kan worden en ii) een tweede probe die gelabeld is met digoxigenine, die vervolgens kan hybridiseren met het enkelstrengs DNA aangrenzend aan de gehybridiseerde eerste probe; c. het biotine-label van de eerste probe die gehybridiseerd is aan het enkelstrengs DNA vervolgens te binden aan de streptavidine; d. bij een perfecte hybridisatie tussen beide probes en het doelwit DNA verbindt een ligase- enzym beide probes; e. de geligeerde probes vervolgens te denatureren van het gehybridiseerde enkelstrengs DNA; f. de niet-gebonden gelabelde probe wordt vervolgens

weggewassen, waarna g. de gebonden digoxigenine vervolgens gedetecteerd kan worden.

Dit geeft uiteindelijk weer of een specifieke nucleotide-verandering voorkomt in een dubbelstrengs DNA fragment.

Solutie hybridisatie vormt een essentiële stap bij sequentie-analyse en sommige puntmutatie detectie methoden. In de praktijk wordt deze hybridisatie vaak belemmerd waardoor zuivering van geamplificeerde producten noodzakelijk is.

Hoofdstuk Drie beschrijft het effect van voorbehandeling van exonuclease op verschillende puntmutatie detectie methoden. Tevens worden de verschillende stappen in de Point-EXACCT procedure in detail besproken. Door gebruik van het enzym exonuclease kunnen heterozygote varianten gedetecteerd worden met behulp van fluorescentie sequentie-analyse. Door exonuclease heeft Point-EXACCT een hoge sensitiviteit en kan deze methode in potentie gebruikt worden voor de detectie van genetische veranderingen die reeds sporadisch voorkomen tussen 'genetisch normale' cellen.

Hoofdstuk Vier beschrijft de validering van de Point-EXACCT methode in een grote groep patiënten met een niet-kleincellig longcarcinoom. De resultaten van de Point-EXACCT methode worden hier vergeleken met twee sequentie-analyse methoden. De met sequencing aangetoonde mutaties zijn eveneens met Point-EXACCT gedetecteerd. Bovendien zijn er meer mutaties gevonden met Point-EXACCT dan met beide andere methoden. Tevens zijn K-ras puntmutaties aangetoond bij patiënten met een plaveiselcelcarcinoom. Dit wordt verklaard door de hoge sensitiviteit van de Point-EXACCT methode. Al met al tonen de resultaten dat Point-EXACCT op zijn minst beter is dan de beide sequentie-analyse methoden.

Hoofdstuk Vijf bestudeert de rol van het K-ras gen als vroege marker bij longkanker. Dit wordt aangetoond aan de hand van K-ras puntmutatie detectie in sputum van patiënten met een longtumor. De aanwezigheid K-ras puntmutaties is onderzocht in DNA geïsoleerd van paraffine-materiaal van zowel tumor als sputum monsters van 22 patiënten met een adenocarcinoom van de long. In meer dan de helft van de tumoren is een K-ras puntmutatie gevonden, en in 45% van de patiënten met een K-ras positieve tumor is dezelfde K-ras mutatie aangetoond in tenminste één sputum monster. Er zijn geen K-ras puntmutaties gedetecteerd in sputum van patiënten met een K-ras negatieve tumor. De tijd tussen de detectie van een K-ras puntmutatie in sputum en de uiteindelijke diagnose van longkanker varieerde tussen 1 maand en bijna 4 jaar. Uit deze studie van archief materiaal blijkt dat de Point-EXACCT methode bruikbaar is voor de detectie van K-ras puntmutaties in sputum van patiënten met een adenocarcinoom van de long. Point-EXACCT kan een belangrijke bijdrage leveren aan de vroegdetectie van longkanker aangezien K-ras puntmutaties gedetecteerd zijn in sputum waarin er met cytologisch onderzoek geen aanwijzingen voor maligniteit werden aangetroffen.

In **hoofdstuk Zes** wordt bestudeerd of K-ras puntmutaties ook voorkomen in niet-maligne bronchusepitheel. Bij 75 patiënten, die chirurgisch behandeld zijn voor een niet-kleincellig longcarcinoom, is bronchusepitheel geanalyseerd op de aanwezigheid van K-ras puntmutaties. K-ras puntmutatie detectie is zowel uitgevoerd op tumormateriaal als op normale bronchiale brushes, die verzameld zijn op enige afstand van de tumorlokatie. K-ras puntmutaties worden enkel in tumorweefsel aangetoond maar niet in het normale bronchusepitheel. Dit steunt de hypothese dat het optreden van K-ras puntmutaties specifiek gerelateerd is aan tumorvorming en dus laat in de carcinogenese optreedt.

Hoofdstuk Zeven bestudeert de toegevoegde diagnostische waarde van K-ras puntmutaties in bronchusspoelsels van patiënten verdacht voor een longtumor. Een serie bronchusspoelsels, waarvan de diagnose maligniteit met behulp van cytologie niet kon worden gesteld, is onderzocht met de Point-EXACCT methode. Deze bronchusspoelsels zijn afgenomen bij patiënten met een perifere schaduw op de röntgenfoto. Tevens is er een controle-groep bestudeerd. Puntmutatie detectie in codon 12 van het K-ras gen is uitgevoerd op bronchusspoelsels en tumormateriaal. Analyse met behulp van Point-EXACCT leverde in 21% van de bronchusspoelsels een K-ras puntmutatie op. Puntmutaties zijn niet gedetecteerd in de bronchusspoelsels van de controle-groep. De aanwezigheid van K-ras puntmutaties in bronchusspoelsels uit een kwab met een perifere schaduw op de long rechtvaardigt een curatieve ingreep. Moleculaire analyse kan dus een belangrijke bijdrage leveren aan de diagnostische bronchoscope procedure.

Hoofdstuk Acht bepaalt de prognostische waarde van specifieke K-ras genotypen bij patiënten met een niet-kleincellig longcarcinoom. Associaties van K-ras puntmutaties met klinische kenmerken en overleving zijn bestudeerd. De overlevingscurves tonen een prognostisch significante rol van K-ras puntmutaties op de ziekte-vrije overleving en een nadelig effect van K-ras puntmutatie op de algemene overleving. Bij een verandering van de wildtype sequentie GGT (coderend voor glycine) naar GAT (coderend voor aspartaat) en ATT (coderend voor isoleucine) is een significant slechtere overleving gevonden, vergeleken met het wildtype of overige K-ras genotypen. Hierbij suggeren wij dat patiënten met een slechtere prognose geïdentificeerd kunnen worden op basis van specifieke K-ras codon 12 mutaties. Op basis van deze data en een literatuuroverzicht menen wij dat deze patiënten in aanmerking kunnen komen voor adjuvante therapie.

Hoofdstuk Negen geeft de technische aspecten van de Point-EXACCT methode weer. Mogelijke beperkingen en verbeteringen worden vervolgens bediscussieerd. Daarbij worden suggesties voor vroegdetectie van longkanker gedaan.

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Curriculum Vitae

De schrijfster van dit proefschrift werd geboren op 11 mei 1972 te Bilzen (België). In 1990 behaalde zij het 'Getuigschrift van Hoger Secundair Onderwijs' en het 'Bekwaamheidsdiploma dat toegang verleent tot het Hoger Onderwijs' aan de Onbevleete Ontvanging te Tongeren (België). In datzelfde jaar begon zij met de studie Gezondheidswetenschappen aan de Universiteit Maastricht, met als afstudeerrichting Biologische Gezondheidkunde. Het doctoraalexamen werd behaald op 31 augustus 1994 na een stage aan de afdeling Pathologie. Daar werd het onderzoek verricht waarvan dit proefschrift de weerslag is. Vanaf 1 september 1994 begon zij voor 2 jaar als onderzoeksassistente bij de afdeling Pathologie, waar zij vanaf 1 juli 1996 was aangesteld als assistent in opleiding (AIO). Het onderzoeksproject beschreven in het voorliggende proefschrift, gesubsidieerd door de Wetenschappelijke Adviesraad voor Roken en Gezondheid, is op 31 augustus 1998 afgerond. Voor haar werk in hoofdstuk 5 ontving zij een 'Junior Investigator Award' op de '8th World Conference on Lung Cancer' in 1997. Vanaf 1 september 1998 is zij aangesteld als Post-doc bij de afdeling Pathologie van de Universiteit Maastricht.

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